REMARKS

I. Status Summary

Claims 1-6, 8-11, 16-25, 27-30, 33-39, and 41-44 are pending in the present application. Claims 3, 6, 8-11, 17, 22, 25, 27-30, 33-39, and 41-44 currently stand withdrawn. Claims 1, 2, 4, 5, 16, 18-21, 23, and 24 have been examined by the United States Patent and Trademark Office (hereinafter "the Patent Office") and presently stand rejected.

Claims 1, 4, 18, and 23 have been amended. Support for the amendments can be found throughout the claims and specification as filed, including at page 6, lines 14-17; at page 18, lines 7-9; and in Figures 3A-3C and 6A-6B. Thus, no new matter has been added by the amendments to the claims.

Reconsideration of the application as amended and in view of the remarks presented herein below is respectfully requested.

II. Response to Rejections under 35 U.S.C. §112, Second Paragraph

Claims 1, 2, 4, 5, 16, 18-21, 23, and 24 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. More particularly, the Patent Office contends that claims 1 and 18 are indefinite because the recitation "without compromising the function of the antigen" is unclear. The Patent Office further contends that claims 5 and 24 lack antecedent basis in claims 1 and 18, respectively, because ovalbumin is not a plasma protein.

After careful consideration of the rejection and of the Patent Office's comments, applicants respectfully traverse the rejection and offer the following remarks.

Initially, applicants respectfully submit that claim 1 has been amended to recite "in a mammal in need thereof, wherein said mammal expresses a Fcγ receptor" and "so as to form an antibody-antigen conjugate, wherein said antigen is a protein substantially soluble *in vivo*, and wherein said antibody-antigen conjugate treats immune thrombocytopenia or inflammatory arthritis of the mammal expressing the Fcγ receptor." Support for the amendment can be found in claim 1 as originally filed. Further support for protein antigens that are soluble *in vivo* can be found in the instant specification at,

for example, page 6, lines 14-17. Support with regard to expression of the Fcγ receptor can be found, for example in Figures 6A and 6B and at page 18, lines 7-9.

Claim 4 has been amended to recite "the antibody-antigen conjugate prior to administering said conjugate." Support for the amendment can be found in claim 4 as originally filed.

Claim 18 has been amended to replace "patient" with "mammal in need thereof, wherein said mammal expresses a Fcγ receptor" or "mammal." Claim 18 has also been amended to recite "so as to form an antibody-antigen conjugate in said mammal, wherein said antigen is a protein substantially soluble *in vivo*, and wherein said antibody-antigen conjugate inhibits platelet clearance in the mammal expressing the Fcγ receptor." Support for the amendment can be found in claim 18 as originally filed and in the instant specification, for example, at page 2, lines 28-30; at page 6, lines 14-17; and at page 18, lines 7-9. Additional support can be found in Figures 3A-3C, 6A, and 6B.

Claim 23 has been amended to recite "to form the antibody-antigen conjugate prior to administering said conjugate to said mammal." Support for the amendment can be found in claim 23 as originally filed.

Further support with regard to the amendments can be found in the priority documents, i.e., U.S. Provisional Patent Application Nos. 60/558,080 and 60/613,712. See for example, paragraphs [0019] and [0022] and Figures 6A and 6B of U.S. Provisional Patent Application No. 60/558,080, as well as paragraphs [0020] and [0025] and Figures 6A and 6B of U.S. Provisional Patent Application No. 60/613,712.

Applicants respectfully submit that, as described in Manual of Patent Examining Procedure (hereinafter "MPEP") § 2173.02, the definiteness of claim language must be analyzed, not in a vacuum, but in light of (a) the content of the particular application disclosure; (b) the teachings of the prior art; and (c) the claim interpretation that would be given by one possessing the ordinary level of skill in the pertinent art at the time the invention was made. The claim must be considered as a whole. Id. Further, a claim term that is not used or defined in the specification is not indefinite if the meaning of the term is discernible. Id., citing *Bancorp Services, L.L.C. v. Hartford Life Ins. Co.*, 359 F.3d 1367, 1372, 69 USPQ2d 1996, 1999-200 (Fed. Cir. 2004). The test for definiteness under 35 U.S.C. § 112, second paragraph, is whether "those skilled in the

art would understand what is claimed when the claim is read in light of the specification." Id., citing *Orthokinetics, Inc. v. Safety Travel Chairs, Inc.*, 806 F.2d 1565, 1576, 1 USPQ2d 1081, 1088 (Fed. Cir. 1986).

Applicants respectfully submit that the phrase "without compromising the function of the antigen" would be clearly understood by those of skill in the art in the context of either claim 1 or 18 as a whole, particularly in light of the instant specification. Indeed, applicants respectfully submit that the skilled artisan, in reviewing the instant specification and claims, would be able to interpret the phrase as referring, for example, to the known ability of certain antibodies to be "neutralizing" or "non-neutralizing." More particularly, it is known that "neutralizing antibodies" can bind to particular epitopes on an antigen to mask a known biological/biochemical function of that antigen (e.g., receptor activity, regulatory activity, infectivity, etc.). Alternatively, "non-neutralizing" antibodies can bind to epitopes of an antigen and not interfere with known biological/biochemical activity. See for example, U.S. Patent No. 4,853,326, attached hereto as **Exhibit A**, column 1, line 61 to column 2, line 2 and U.S. Patent Application Publication No. 2007/0202099, attached hereto as **Exhibit B**, at paragraph [0011]. Applicants respectfully note that Mostböck (*Current Pharmaceutical Design*, 2009, 15, 809-825; hereinafter "Mostböck"), cited by the Patent Office in the Offical Action, also uses the terms "neutralizing and "non-neutralizing." See for example, Mostböck, page 812, the paragraph under the heading "IL-2" describing non-neutralizing auto-IL-2 antibodies.

Further, applicants respectfully submit that the instant specification describes not only that antibody binding does not compromise the function of the antigen, it also recites that the antigen can be a non-functional/inert antigen. See Instant Specification, page 3, line 7. The use of the words "antigen" and "binding," as well as the recited formation of an "antibody-antigen conjugate" even from a possible "non-functional" antigen clearly indicates that the "function" of the antigen being addressed by the phrase at issue is a function other than antigenicity of the protein antigen for the antibodies of the instant methods. Moreover, the phrase "without compromising the function of the antigen" recited in the instant claims would not be interpreted as precluding the antibody-antigen conjugate that is formed from having a new function as

a conjugate, such as the instantly claimed ability to treat ITP or inflammatory arthritis. Rather, the instantly claimed methods are based on a particular activity of the antibody/antigen conjugate that is distinct from abolishing the biological activity that the antigen has alone, e.g., by binding a particular receptor *in vivo*. In other words, one of ordinary skill in the art would recognize that the phrase "without compromising the function of the antigen" is intended to indicate that the presently claimed methods are effected by a mechanism other than the elimination or sequestration of disease-causing or disease-regulating protein ligands by antibodies. Accordingly, applicants respectfully submit that the Patent Office's comments regarding "without compromising the function of the antigen" have been addressed. Applicants respectfully submit that claims 1 and 18 meet the requirements of 35 U.S.C. § 112, second paragraph.

The phrase "plasma protein" has also been deleted from claims 1 and 18. Claims 1 and 18 now recite "said antigen is a protein substantially soluble *in vivo*." Applicants respectfully submit that the one of ordinary skill in the art would appreciate that ovalbumin and other foreign protein antigens can be substantially soluble *in vivo* in a mammal. Therefore, applicants respectfully submit that the Patent Office's remarks regarding the lack of antecedent support for ovalbumin in claims 5 and 24 have been rendered moot.

Applicants respectfully believe that the other claims, claims 2, 4, 16, 19-21, and 23 were included in the instant rejection due to their dependency from claim 1. Thus, in view of the remarks above regarding claims 1 and 18, applicants respectfully submit that claims 2, 4, 16, 19-21, and 23 are also believed to meet the requirements of 35 U.S.C. § 112, second paragraph.

Applicants respectfully request that the rejection of claims 1, 2, 4, 5, 16, 18-21, 23, and 24 under 35 U.S.C. § 112, second paragraph, be withdrawn and further request that claims 1, 2, 4, 5, 16, 18-21, 23 and 24 be allowed at this time.

III. Response to Rejections under 35 U.S.C. §112, First Paragraph, The Enablement Requirement

Claims 1, 2, 4, 5, 16, 18-21, 23, and 24 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement.

The Patent Office contends that the specification is not enabling for a method reciting "by means of an *in vivo* antibody antigen interaction without compromising the function of the antigen." The Patent Office alleges that this particular phrase can be interpreted as including compromising the ability of the molecule to perform its native biologic function, which the Patent Office contends can include the ability to function as an antigen. Further the Patent Office contends that claims 5 and 24 fail to meet the enablement requirement because ovalbumin is not a plasma protein as recited in the claims from which claims 5 and 24 depend.

After careful consideration of the rejection and of the Patent Office's comments, applicants respectfully traverse the rejection and offer the following remarks.

Initially, as described hereinabove, claims 1 and 18 have been amended to delete the phrase "plasma protein." Claims 1 and 18 currently recite "wherein said antigen is a protein substantially soluble *in vivo*." Further, claim 1 has been amended to recite "a mammal in need thereof, wherein said mammal expresses a Fcγ receptor" and "wherein said antibody-antigen conjugate treats immune thrombocytopenia or inflammatory arthritis of the mammal expressing the Fcγ receptor." Claim 18 has been amended to recite "a mammal in need thereof, wherein said mammal expresses a Fcγ receptor" and "wherein said antibody-antigen conjugate inhibits platelet clearance in the mammal expressing the Fcγ receptor."

In general, applicants respectfully submit that the presently disclosed and claimed subject matter relates to methods for treating immune thrombocytopenia (ITP) or inflammatory arthritis comprising administering to a mammal expressing a Fcγ receptor an antibody and/or a complementary antigen that is soluble *in vivo* to form an antibody-antigen conjugate. For example, the antigen-antibody conjugate can inhibit platelet clearance (see Instant Specification, page 2, lines 28-32; and Figures 3A-3C) associated with ITP. As further described in the instant specification, ITP-related platelet clearance can be mediated by Fcγ receptor-bearing macrophages in the reticuloendothelial system (RES). See Instant Specification, page 1, lines 9-14. In this context, the instant specification provides data showing that administration of anti-ovalbumin antibodies and ovalbumin treats ITP in mice that express FcγRIIB, but does not treat mice that do not express FcγRIIB. See Instant Specification, page 18, lines 7-9

and Figures 6A and 6B. See also, Instant Specification, page 22, lines 21-25. Similar results are described for treatments with anti-albumin and anti-transferrin antibodies (which can react with serum albumin and transferrin already present in a mammal). See Instant Specification, page 19, lines 10-15 and Figure 8B. Accordingly, it is believed that the instant specification provides sufficient guidance to the skilled artisan to show that antibodies of various unrelated soluble protein antigens can be used to form antibody-antigen conjugates that can treat ITP in mammals that express Fcγ receptor. The instant specification further provides data related to the treatment of inflammatory arthritis. See Instant Specification, page 21, lines 4-25 and Figures 10A and 10B.

With regard to the Patent Office's comments concerning the phrase "without compromising the function or the antigen", applicants respectfully submit that, as described hereinabove, it is believed that such antibodies are known in the art. For example, the art recognizes the ability of certain antibodies to be "neutralizing" or "non-neutralizing." Moreover, applicants respectfully submit that the teachings of Mostböck referred to by the Patent Office in support of the instant rejection relate to particular anti-cytokine antibodies. Mostböck does not teach or suggest that it is impossible for some antibodies to bind to their antigens without compromising the function of the antigen. Indeed, Mostböck itself describes certain antibodies as being "non-neutralizing". See for example, Mostböck, page 812, the paragraph under the heading "IL-2" describing non-neutralizing auto-IL-2 antibodies.

It is further believed that, that in the context of claims 1 and 18 as a whole, particularly in view of the specification, one of ordinary skill in the art would appreciate that the phrase "without compromising the function of the antigen" is not intended to imply that the antigen is incapable of functioning as an antigen, i.e., could not form the claimed antibody-antigen conjugate. Rather, the skilled artisan would appreciate that the phrase relates to biological functioning of the antigen that might involve, for example, to receptor binding, infectivity, or other disease-modifying behavior. Thus, the phrase is intended to help describe that the instant antibody-antigen conjugates do not effect treatment of ITP or inflammatory arthritis by masking an ITP or arthritis-causing activity of the antigen.

Therefore, when "without compromising the function of the antigen" is viewed with the meaning that one of ordinary skill in the art would give to the phrase, and particularly in view of the aforementioned data described in the instant specification with regard to the use of three unrelated anti-soluble protein antibodies, i.e., anti-ovalbumin, anti-albumin, and anti-transferrin, applicants respectfully submit that the instant specification provides sufficient guidance for the skilled artisan to practice the methods of claims 1 and 18 without undue experimentation.

Turning now to the Patent Office's comments regarding claims 5 and 24 and the terms "plasma protein" and "ovalbumin," applicants respectfully submit that these comments have been rendered moot by the claim amendments. In addition, as noted above, applicants respectfully submit that the instant specification provides particular support that ovalbumin is a suitable antigen for use according to the presently claimed methods, for example, as shown in Figure 6A. Also, applicants respectfully submit that one of ordinary skill in the art would appreciate that ovalbumin is an antigen that is soluble *in vivo* in a mammal.

Thus, applicants respectfully submit that claims 1, 5, 18, and 24 meet the enablement requirement of 35 U.S.C. § 112, first paragraph. It is respectfully believed that claims 2, 4, 16, 19-21, and 23 were included in the rejection due to their dependence from claims 1 and 18. Since claims 1 and 18 are believed to meet the enablement requirement, it is further believed that dependent claims 2, 4, 16, 19-21, and 23 also meet the enablement requirement.

Accordingly, applicants respectfully request that the rejection of claims 1, 2, 4, 5, 16, 18-21, 23, and 24 under 35 U.S.C. § 112, first paragraph, the enablement requirement, be withdrawn. Applicants further respectfully request that claims 1, 2, 4, 5, 16, 18-21, 23, and 24 be allowed at this time.

IV. Response to Rejections under 35 U.S.C. §112, First Paragraph, The Written Description Requirement

Claims 1, 2, 4, 5, 16, 18-21, 23, and 24 have been rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. The Patent Office contends that there is no support in the specification as

originally filed for the recitation "plasma protein" in claims 1 and 18. The Patent Office further contends that there is no support for the use of foreign plasma proteins as per claim 2. Thus, the Patent Office contends that the claimed invention constitutes new matter. The rejection is respectfully traversed.

Initially, as described hereinabove, claims 1 and 18 have been amended to delete the phrase "plasma protein" and to recite "wherein said antigen is a protein substantially soluble *in vivo*." Support for the amendment can be found in claims 1 and 18 as originally filed and in the instant specification, for example, at page 6, lines 14-17. Support can also be found in the priority documents, U.S. Provisional Patent Application No. 60/558,080 (at paragraph [0022]) and U.S. Provisional Patent Application No. 60/613,712 (at paragraph [0025]). The instant specification further provides particular description regarding the use of three unrelated soluble proteins antigens, i.e., ovalbumin, albumin, and transferrin. See Instant Specification, for example, page 18, lines 7-9; page 19, lines 10-15; page 22, lines 21-25; and Figures 6A, 6B, and 8B. Applicants respectfully submit that ovalbumin is a foreign antigen in mammals.

According, applicants respectfully submit that the Patent Office's remarks regarding "plasma proteins" and new matter have been rendered moot. Applicants further respectfully submit that claims 1 and 18, as well as claims 2 and 21, which recite foreign antigens, meet the written description requirement of 35 U.S.C. § 112, first paragraph.

Applicants respectfully believe that claims 4, 5, 16, 19, 20, 23 and 24 were included in the instant rejection in view of their dependence from claims 1 and 18. Accordingly, since claims 1 and 18 are believed to meet the written description requirement, it is further respectfully believed that claims 4, 5, 16, 19, 20, 23, and 24 also meet the written description requirement.

Applicants respectfully request that the rejection of claims 1, 2, 4, 5, 16, 18-21, 23, and 24 under 35 U.S.C. § 112, first paragraph, the written description requirement, be withdrawn and further ask that claims 1, 2, 4, 5, 16, 18-21, 23, and 24 be allowed at this time.

V. Response to Comments Regarding Siragam

At page 6 of the Official Action, the Patent Office notes that the prior rejection under 35 U.S.C. § 102(b) over Siragam et al. (hereinafter "Siragam") was withdrawn in view of the amended claims and because ovalbumin is not a plasma protein.

For clarity of the record, and in the event that the Patent Office might consider reapplying Siragam in view of the deletion of "plasma protein", applicants respectfully note that the presently presented claims are believed to be fully supported by PCT/CA2005/000472, filed March 30, 2005. The claims are also believed to be fully supported by the priority documents, i.e., U.S. Provisional Patent Applications Nos. 60/558,080 and 60/613,712, filed March 30, 2004 and September 29, 2004. Accordingly, applicants respectfully submit that Siragam is not believed to be prior art to the instant application under either 35 U.S.C. § 102(b) or 35 U.S.C. § 102(a).

CONCLUSION

In light of the above amendments and remarks, it is respectfully submitted that the present application is now in condition for allowance, and an early notice to such effect is earnestly solicited.

If any small matter should remain outstanding after the Patent Examiner has had an opportunity to review the above Remarks, the Patent Examiner is respectfully requested to telephone the undersigned patent attorney in order to resolve these matters and avoid the issuance of another Official Action.

DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any fees associated with the filing of this correspondence to Deposit Account No. **50-0426**.

Respectfully submitted,

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Enclosures: **Exhibits A-B**

EXHIBIT A

United States Patent [19]

Quash et al.

[11] **Patent Number: 4,853,326**

[45] **Date of Patent: Aug. 1, 1989**

[54] **CARBOHYDRATE PERTURBATIONS OF VIRUSES OR VIRAL ANTIGENS AND UTILIZATION FOR DIAGNOSTIC PROPHYLACTIC AND/OR THERAPEUTIC APPLICATIONS**

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[21] Appl. No.: **928,631**

[22] Filed: **Nov. 18, 1986**

[30] **Foreign Application Priority Data**

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[51] **Int. Cl.**[4] **G01N 33/531**; G01N 33/569; G01N 33/571; G01N 33/576

[52] **U.S. Cl.** **435/5**; 435/7; 436/507; 436/510; 436/511; 436/518; 436/543; 436/548; 436/812; 436/820

[58] **Field of Search** 435/5, 7; 436/507, 812, 436/820, 510, 511, 543, 548

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,195,265	4/1980	Koprowski	424/85
4,217,338	8/1980	Quash	435/543
4,355,102	10/1982	Quash	435/5
4,367,309	1/1983	Kondo et al.	424/85
4,419,444	12/1983	Quash	435/7
4,604,348	8/1986	Neurath	436/518
4,634,666	1/1987	Englemann et al.	435/68

FOREIGN PATENT DOCUMENTS

0088695	9/1983	European Pat. Off. .
0117657	5/1984	European Pat. Off. .
0122841	10/1984	European Pat. Off. .
0156559	9/1982	Japan .
84/04327	11/1984	World Int. Prop. O. .
86/03224	6/1986	World Int. Prop. O. .

OTHER PUBLICATIONS

Glorioso et al, *Virology*, 126, 1–16, 1983.
Berman et al, *Trends in Biochem*, 3, 51–53, 1985.
Finegold et al, *Bailey and Scott's Diagnostic Microbiology*, 5th Ed., C. V. Mosby Company, Saint Louis, 1978, pp. 272–278.
Dreesman et al, *Virology*, 69, 700–709, 1976.
Hagenaars et al, *Journ. Virol. Meth.*, 6, 233–239, 1983.
Krech et al, *Z. Immun.-Forsch., Bd.*, 141, 411–429, 1971.
Lee et al, *Prod. Natl. Acad. Sci.*, 81, 3856–3860, 1984.
Peake et al, *Chem. Abstr.*, 96, 214032t, 1982.
Bosch et al, *Chem. Abstr.*, 96, 214039a, 1982.
Quash, *Chem. Abstr.*, 90, 148119z, 1979.
Wang et al, *Proc. Natl. Acad. Sci.*, 83, 6159–6163, 1986.
Elder et al, *Journ. of Virology*, 57, 340–342, 1986.

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[57] **ABSTRACT**

Novel and improved methods for diagnosis, prognosis, prophylaxis and therapy of viral infections are described. The novel methods employ a virus, viral antigen or fragment thereof in which "perturbation" of an oligosaccharide moiety renders the virus, viral antigen or fragment thereof more specifically recognizable or reactive with neutralizing antibody. As described, "perturbation" of an oligosaccharide moiety encompasses any modification that (1) alters the chemical or physical structure of a carbohydrate residue that is naturally present; (2) that removes, wholly or in part, a carbohydrate residue; and/or (3) that prevents or alters addition of a carbohydrate residue. A variety of methods for oligosaccharide "perpetuation" are also described.

18 Claims, No Drawings

CARBOHYDRATE PERTURBATIONS OF VIRUSES OR VIRAL ANTIGENS AND UTILIZATION FOR DIAGNOSTIC PROPHYLACTIC AND/OR THERAPEUTIC APPLICATIONS

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1. FIELD OF THE INVENTION

The present invention relates generally to novel and improved methods for diagnosis, prophylaxis and therapy of viral infections. More particularly the invention relates to novel methods employing a virus, viral antigen or fragment thereof in which an oligosaccharide moiety is perturbed in such a way that the virus, viral antigen or fragment thereof is specifically recognized by or reacts specifically with neutralizing antibodies. The term "perturbed" oligosaccharide moiety is intended to encompass any modification (1) that alters the chemical or physical structure of a carbohydrate residue that is naturally present; (2) that removes, wholly or in part, a carbohydrate residue that is naturally present; and/or (3) that prevents or alters the addition of a carbohydrate residue (i.e. prevents or alters glycosylation).

The perturbed viruses, viral antigens and fragments thereof prepared according to the present invention are useful for in vitro diagnostic and prognostic applications as well as for in vivo prophylactic and therapeutic applications.

2 BACKGROUND OF THE INVENTION

Viruses are important etiological agents of a wide variety of diseases. In animals the immune response comprises one of the basic mechanisms to fight viral infections. Classically, the immune response encompasses two facets: the B-lymphocyte antibody response, referred to as humoral immunity and a T-lymphocyte-mediated response, known as cell-mediated immunity. The present application is concerned particularly with the antibody response.

While specific antibody of classes IgG, IgM and IgA can bind to any accessible epitope on a surface protein of a virion, only those antibodies which bind with reasonably high avidity to particular epitopes on a particular protein in the outer capsid or envelope are capable of neutralizing the infectivity of the virion. These are termed "neutralizing antibodies." "Neutralization" as used throughout the instant specification is intended to include not only (1) classical virus neutralization which results when antibody binds to a surface antigen of a virion which ordinarily binds to a receptor on the surface of a susceptible cell and thereby prevents infection of a susceptible cell or leads to opsonization but also includes (2) interactions such as the binding of an antibody to neuraminidase of influenza virus (IF) which results in inhibition of release of progeny virus particles from the plasma membrane of infected cells and slows virus spread and (3) binding of an antibody to fusion protein (F) of paramyxoviruses which does not prevent initiation of infection but does block the direct cell to cell spread of newly formed virions once infection has been established. Antibodies directed against irrelevant or inaccessible epitopes of surface proteins, or against internal proteins of the virion, or virus-coded nonstructural proteins, such as virus-encoded enzymes can sometimes exert indirect immunopathological effects, but may play no role in elimination of the infection. These are "non-neutralizing antibodies." In fact, certain non-neutralizing antibodies not only form damaging circulating "immune complexes" but may actually impede access of neutralizing antibody and enhance the infectivity of the virion for some cells. For example, in the presence of sub-neutralizing concentration of neutralizing antibody or excess of non-neutralizing antibodies viruses such as togaviruses are actually taken up more efficiently by macrophages (via Fc receptors on the macrophage to which the virus-antibody complex binds). The virus multiples intracellulary to high titer inside the macrophages. Hence the non-neutralizing antibodies act as "enhancing antibody." Specific examples of such viruses include dengue virus types 1–4.

Thus in response to a viral infection, two very different kinds of antibodies are produced: neutralizing antibodies and non-neutralizing antibodies. Each is present in the serum of infected individuals or individuals previously exposed to a virus or a viral antigen in varying amounts. In order to assess the true immunocompetent status of an individual it is necessary to know the absolute and relative amounts of both neutralizing and non-neutralizing antibodies. Yet conventional serological assays of antiviral antibodies do not, and in fact cannot, distinguish these two kinds of antibodies. Conventional serological assays measure the presence of both types of antibodies. Hence there is no serological method for measuring or assessing the true immune status or immunocompetence of individuals.

The only conventional method for assessing virus neutralizing ability of serum of individuals has been the virus neutralization assay such as that described by Krech et al., Z. Immuno., Forsch. Bd. 141 S: 411–29 (1971). Neutralization assays require: (1) use of infectious virus and (2) cell culture techniques. Such assays are slow, cumbersome, labor intensive and expensive. Hence there has been a long-felt need for a rapid, inexpensive accurate serological method to assess the immunocompetent status of individuals.

Examples of specific situations in which a rapid, easy test for assessing the immunocompetence of an individual is particularly important include, but are not limited to, the following. Firstly, exposure of a pregnant female to a virus such as rubella virus or cytomegolovirus poses significant risk of congenital defects for the fetus. Using conventional serological methods such as ELISA assays the titer of all IgM and IgG antibodies against the relevant virus, both neutralizing and non-neutralizing.

may be determined. If the total IgM level is elevated indicating that the response is due to reaction by a presumably "naive" immune system, a therapeutic abortion will be recommended because it is unlikely that neutralizing antibodies against the virus are present. If, however, only the total IgG level is elevated, no therapeutic abortion will be recommended because the test cannot distingush whether the IgG's present are neutralizing or non-neutralizing antibodies. The patient is faced with a long stress-filled pregnancy which may end in a child with congenital defects. Secondly, exposure of (or reactivation of previous infection associated with immunosuppression) organ transplant or bone marrow transplant patients to viruses such as cytomegalovirus (CMV) poses significant risks of clinical disease states including pneumonia, hepatitis, retinitis, encephalitis, etc. Moreover, the glomerulopathy induced by CMV adversely affects the survival of kidney grafts, so that renal transplant patients face additional life-threatening organ rejection [see generally, White et al., eds., in Medical Virology, 3d ed., Academic Press, Inc., New York, pp. 419–426 (1986)]. Thirdly, viral infections pose significant, indeed often life-threatening risks for immuno-suppressed patients including cancer patients undergoing chemotherapy, and those afflicted with either congenital or acquired immunodeficiency such as acquired immune deficiency syndrome (AIDS). Fourthly, certain viral infections endemic to specific geographic areas pose significant risks, for example, for military or diplomatic personnel stationed in these areas. Specific examples include but are not limited to Rift Valley fever, dengue etc. Vaccines may protect by actively eliciting the production of neutralizing antibodies. Evaluation of the immunocompetent status of personnel to be sent to these areas following vaccination is important.

In all the above examples, there is a need for rapid, serological methods for determining both the presence and titer of virus neutralizing antibodies. Examples of formats useful in such rapid, serological methods include but are not limited to Enzyme-Linked Immunosorbent Assays (ELISA), radioimmunoassays (RIA), immunofluoresence or other fluorescence-based assays, agglutination assays, etc.

Hagenaars et al., J. Virol. Methods 6: 233–39 (1983) described a modified inhibition ELISA assay which showed some correlation between ELISA titers and neutralization assay titers for polio virus type I. Unlike the presently described assays, however, the modified inhibition ELISA of Hagenaars et al. was more complex and cumbersome.

3. SUMMARY OF THE INVENTION

The present invention is based upon the surprising discovery that whole viruses, viral antigens and fragments thereof in which the structure of an oligosaccharide moiety of a viral glycoprotein has been "perturbed" are recognized more efficiently by serum, plasma or immunoglobulin fractions containing neutralizing antibody molecules. As used throughout the instant specification, the terms "perturbed" oligosaccharide and oligosaccharide "perturbation" are intended to encompass any modification (1) that alters the chemical or physical structure of a naturally occurring carbohydrate residue; (2) that removes, wholly or in part, a naturally occurring carbohydrate residue; and/or (3) that prevents or alters the addition of a carbohydrate residue to a virus, viral antigen of fragment thereof. Any techniques known in the art for achieving such oligosaccharide "perturbations" including, but not limited to genetic engineering methods, are intended to fall within the scope of the present invention.

Based on this discovery, one embodiment of the present invention provides a novel method for detecting, as well as quantitating, the presence of virus neutralizing antibodies in samples of body fluids such as serum, plasma, various immunoglobulin fractions, etc. The novel method of the invention has the following advantages over conventional assays for neutralizing antibodies: (1) does not require use of cell culture techniques; (2) does not require use of infectious virus; (3) comprises a straight-forward serological assay; and (4) is complete in 4 hours or less. In contrast, conventional assays for virus neutralizing antibodies require: (1) cells in culture; (2) infectious virus; (3) skilled personnel; and (4) 5 days or so before an answer can be obtained. Thus, the present method is faster, easier and less complex than conventional methods. It does not require skilled personnel trained in the handling of infectious viral materials and is safer for use because even trained personnel need not be exposed to infectious virus.

Another embodiment of the present invention provides a novel method for preparing compositions comprising a virus, viral antigen or fragment thereof in which the oligosaccharide moiety is perturbed such that the compositions are useful for eliciting the formation of neutralizing antibodies. Thus these compositions provide vaccine formulations which stimulate an active immune response for prophylaxis of viral infections. For example, according to this embodiment a viral antigen is prepared having a perturbed oligosaccharide moiety and administered as a vaccine formulation to actively elicit the production of protective antibodies.

Another alternate embodiment of the present invention provides a variety of novel methods for preparing or identifying monoclonal or polyclonal neutralizing antibodies which can be administered to confer short-term passive immunity for prophylaxis and/or therapy of viral infections. For example, a perturbed viral antigen is used to identify those monoclonal antibodies prepared by hybridoma techniques which are capable of neutralizing virus. Such antibodies could be administered for prophylactic treatment of persons at risk of developing a particular viral disease.

The present invention may be more fully understood by reference to the following detailed description and examples of specific embodiments.

4. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel methods for diagnosis, prognosis, prophylaxis and therapy useful for a variety of viral infections. All the novel methods are based upon utilization of either whole virus, viral antigens or fragments of viral proteins in which "perturbation" of an oligosaccharide moiety renders the virus, viral antigen or fragment thereof more efficiently recognizable by neutralizing antibodies.

4.1. Oligosaccharide Perturbations

According to the present invention, oligosaccharide "perturbation" encompasses any modification (1) that alters the chemical or physical structure of a naturally occurring carbohydrate residue; (2) that removes, wholly or in part a naturally occurring carbohydrate residue; and/or (3) that prevents or alters the addition

of a carbohydrate residue to a virus, viral antigen or fragment thereof. Illustrative examples of oligosaccharide perturbations include, but are not limited to the following.

Whole viruses, viral antigens or fragments thereof are perturbed by mild oxidation of an oligosaccharide moiety of a viral glycoprotein. For example, chemical oxidation of the oligosaccharide moiety can be accomplished using a variety of oxidizing agents such as periodic acid, paraperiodic acid, sodium metaperiodate, and potassium metaperiodate. Oxidation using such oxidizing agents is carried out by known methods. For a general discussion, see Jackson, in Organic Reactions 2, p. 341 (1944); Bunton, in Oxidation Chemistry, Vol. 1., Wiberg, ed., p. 367, Academic Press, New York (1944). The amount of the oxidizing agent depends on the kind of virus or viral antigen, but generally is used in excess of the amount of oxidizable oligosaccharide. The optimal amount can be determined by routine experimentation. The optimal ranges include: pH from about 4 to 8, a temperature range of about 0° to 37° C., and a reaction period of from about 15 minutes to 12 hours. During oxidation, light is preferably excluded from the reaction mixture in order to prevent over oxidation. Alternatively oxidation is achieved using an enzyme, such as galactose oxidase [Cooper et al., J. Biol. Chem. 234: 445–48 (1959)]. The influence of pH, substrate concentration, buffers and buffer concentration on the enzymatic oxidation are reported in Cooper et al., supra.

Whole viruses, viral antigens, or fragments thereof are perturbed by culturing virus infected cells in the presence of glycosylation inhibitors. For example, infected cells may be cultured in the presence of glycosylation inhibitors including, but not limited to: tunicamycin, streptovirudins, and/or glycosidase inhibitors such as carbohydrate analogs such as 2-deoxy-D-glucose and the like, and castanospermine, norjirimycin, 1-deoxynorjirimycin, bromocenduritol, 1-deoxymannojirimycin, and swainsonine, etc.

Whole viruses, viral antigens or fragments thereof are perturbed by enzymatically removing, either wholly or in part, a naturally occurring oligosaccharide moiety by exposing either virus infected cells in culture or isolated virus particles or fragments thereof to an enzyme which is specific for glycoside residues. Useful enzymes include neuraminidase, endo-beta-N-acetyglucosaminidases and the like.

Perturbation of viral antigens according to the present invention is also accomplished using genetic engineering techniques. For example, a gene encoding a particular viral-glycoprotein or fragment may be cloned and expressed in a bacterial organism. In such case, little to no glycosylation of the expressed viral protein occurs, resulting in a perturbed oligosaccharide. Alternatively, a gene encoding the desired viral glycoprotein or fragment can be cloned and expressed in a eukaryotic organism or cell culture. The eukaryotic organism or cell is then cultured in the presence of a glycosylation inhibitor such as tunicamycin or a glycosidase inhibitor such as castanospermine, norjirimycin, and the like resulting in expression of a protein or fragment having a perturbed oligosaccharide. Yet another alternative is to use site-selective mutagenesis techniques to alter a gene encoding a viral antigen or fragment in such a way as to remove or change the site(s) of glycosylation.

In case-s where the amino acid sequence of a viral glycoprotein or fragment is known, chemical methods of peptide synthesis provide yet another method for the preparation of a viral antigen or fragment thereof with a perturbed oligosaccharide.

The foregoing are merely illustrative examples of methods of oligosaccharide perturbation. Any other techniques known to those of skill in the art are intended to be encompassed by the perturbed oligosaccharide moiety.

The perturbed oligosaccharide of the virus, viral antigen or fragment thereof may be further modified, for example, by reduction with reducing agents such as sodium borohydride, cyanoborohydride and the like or by covalent attachment to a soluble or insoluble carrier or support.

4.2. Applications

The viruses, viral antigens and fragments thereof according to the present invention are advantageously used for methods suited for a number of different applications.

4.2.1. Diagnostic and Prognostic Applications

According to one embodiment of the invention, the virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety is used in a variety of methods for assaying a sample of body fluids such as serum, plasma or partially purified immunoglobulin fractions for the presence and titer of neutralizing antibodies.

The viruses, viral antigens or fragments thereof are used as ligands (antigens) to detect the presence of virus neutralizing antibodies in assay systems including but not limited to systems such as: Enzyme-Linked Immunosorbent Assays (ELISA), radioimmunoassays (RIA), immunofluoresence or other fluorescence-based assays, agglutination assays, etc. Examples of viruses for which neutralizing antibody titers are assayed would include those such as described in Section 4.3.

The titers obtained using assays with conventionally treated viruses, for example, conventional ELISA assays, which measure all antibodies, whether neutralizing or non-neutralizing, do not correlate with the titer determined in conventional virus neutralization assays. On the other hand, the titers obtained using the present viruses, viral antigens or fragments thereof having a perturbed oligosaccharide moiety (hereinafter referred to as a "perturbed antigen") have been found by Applicants to be significantly correlated with the titer of virus neutralizing antibodies, as determined by conventional virus neutralization assays. (See, for example, experimental results presented in Sections 5–7, infra). This may be due to a decrease in the binding of non-neutralizing antibody. Hence assays utilizing the present perturbed antigens are useful for diagnostic and/or prognostic prediction of the immuno-competent status of a patient with respect to a particular virus.

The method of the invention for detecting virus neutralizing antibodies in an aqueous sample comprises:

(a) contacting a ligand which comprises a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety with an aqueous sample suspected of containing virus neutralizing antibodies in an assay system selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, an agglutination assay, and an immunofluorescence assay and the like; and

(b) detecting any reaction with the ligand; in which any reaction with the ligand indicates the presence of

neutralizing antibodies in the sample. When quantitating the virus neutralizing antibodies is desired the method further comprises: comparing any reaction of ligand and antibodies to that of a standard.

4.2.1.1. Dissociation and Reassociation of Immune Complexes

Applicants anticipate that in certain instances, virus or virus fragments may be present in varying amounts in serum or plasma samples. These virus or virus fragments could be bound or associated with neutralizing and non-neutralizing antibodies in immune complexes. Consequently, the titer obtained using the present perturbed antigens in in vitro assays may be artifically low (i.e. false negatives). Hence, according to a further improved embodiment of the present invention, such immune complexes are dissociated prior to performing the diagnostic assays. Dissociation of immune complexes can be accomplished by methods known to those of skill in the art including bnt not limited to: use of chaotropic agents such as perchlorate (ClO_4^-), thiocyanate (SCN^-), etc., denaturing agents such as guanidine hydrochloride, urea, etc. and use of variation of pH, and the like. After dissociation, the liberated antibodies are contacted with perturbed antigens according to the present invention under conditons which permit association or reassociation with neutralizing antibodies.

4.2.2. Prophylactic and Therapeutic Applications

According to another embodiment of the present invention, the viruses, viral antigens and fragments thereof having a perturbed oligosaccharide moiety are used as immunogens in vaccine formulations to stimulate an active immune response in a vaccinated host.

When a whole virus having a perturbed oligosaccharide moiety is used, it is necessary to use either an attenuated or avirulent virus or an inactivated virus. An inactivated virus is obtained by treatment of a virus with various chemicals such as formaldehyde; then an oligosaccharide is perturbed using any of the methods described above in Section 4.1. Alternatively, an inactivated virus having a perturbed oligosaccharide moiety is obtained by chemical oxidation of a virus, for example, using periodic acid as described in Section 4.1. Such attenuated or inactivated viruses having a perturbed oligosaccharide moiety should induce antibodies that are more effective at neutralizing viral infections than conventionally attenuated or inactivated viruses.

Subunit vaccines containing only the necessary and relevant immunogenic material such as capsid glycoproteins of non-enveloped icosohedral viruses or the peplomers (glycoprotein spikes) of enveloped viruses or immunogenic fragments thereof can also be prepared in which the oligosaccharide moiety of the glycoprotein or fragment thereof is perturbed according to the present invention. Subunit vaccines can be prepared by isolating the relevant subunit from highly purified viral fractions or using recombinant DNA technology and perturbing the oligosaccharide moiety of the relevant immunogenic subunit as described in Section 4.1.

The vaccine formulations which stimulate an active immune response for prophylaxis of viral infections can be prepared by mixing the virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety in a carrier suitable for use in vivo. In order to enhance the immunological response of the host, the immunogenic virus, antigen or fragment thereof may be formulated with a suitable adjuvant. Suitable adjuvants include, but are not limited to: aluminum hydroxide, surface active substances, lysolecithin, pluronic polyols, polyanions, peptides including but not limited to muramyl peptides, and oil emulsions.

According to another alternate embodiment, a vaccine formulation to stimulate an active immune response for prophylaxis of viral infections is prepared by coupling a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety to an immunogenic peptide or compound in order to enhance or potentiate the immunological response of the host.

According to yet another embodiment, the vaccine formulation can be prepared as a multivalent vaccine. To this end, a mixture of different viruses, viral antigens or fragments thereof each of which contains a perturbed oligosaccharide moiety and which is capable of eliciting an immune response against a different viral pathogen can be mixed together in one formulation.

Many methods can be used to introduce the vaccine formulations described above into a host. These include, but are not limited to: intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes of administration.

According to another embodiment of the present invention, the viruses, viral antigens and fragments thereof having a perturbed oligosaccharide moiety are used in a variety of methods to prepare neutralizing antibodies which can be administered to confer short-term passive immunity for prophylaxis and/or therapy of viral infections. In one mode of this embodiment, a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety is used as an immunogen in any technique that provides for the production of antibody molecules by continuous cell lines in culture. For example, the present immunogens can be utilized in the hybridoma methods originally developed by Kohler and Milstein, and reviewed by them in Sci. Amer. 243: 66–74 (1980) as well as in the human B-cell hybridoma methods described by Kozbor et al., Immunology Today 4: 72 (1983) and the EBV-hybridoma methods for producing human monoclonal antibodies described by Cole et al., in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77–96 (1985) and the like. The monoclonal antibodies produced provide a readily available, consistent source of neutralizing antiboies specific for relevant virus which can be administered for passive immunization.

This embodiment of the invention encompasses a method for preparing a composition for administration to an animal or a human to confer short-term passive immunity or for prophylaxis or therapy of a viral-induced infection comprising: harvesting monoclonal antibodies produced by a hybridoma cell line formed by fusing a myeloma or hybridoma cell and a cell capable of producing antibody against a virus, viral antigen or fragment thereof having a pertrubed oligosaccharide moiety. It further encompasses a method for preparing a composition for administration to an animal or a human to confer short-term passive immnnity or for prophylaxis or therapy of a viral-induced infection comprising: harvesting monoclonal antibodies produced by a lymphocyte cell line formed by transformation by an EBV virus of a mammalian lymphocyte cell capable of producing antibody against a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety. Additionally it encompasses a method for preparing a composition for administration to an animal or a human to confer short-term passive immunity or for

prophylaxis or therapy of a viral-induced infection comprising: (a) contacting a sample containing anti-viral antibodies with an antigen comprising a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety to form an antibody-antigen complex; (b) separating the antibody-antigen complex from the sample; and (c) dissociating the antibody-antigen complex to obtain a purified antiviral antibody composition.

In another mode of this embodiment, a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety is used in a screening assay to identify those viral-specific monoclonal antibodies which although known in the art have not been recognized as neutralizing antibodies. Hence this method screens for and identifies neutralizing monoclonal antibodies which can then be administered for passive immunization.

In yet another alternative mode of this embodiment of the present invention, a virus, viral antigen or fragment thereof is used to prepare neutralizing antibodies from serum, plasma or fractions of immunoglobulins derived therefrom. For example, a virus, viral antigen or fragment having a perturbed oligosaccharide moiety is immobilized and used in a preparative affinity chromatography format to isolate relevant neutralizing antibodies from serum or plasma by the formation of immune complexes. The polyclonal neutralizing antibodies are then separated from the immune complexes by conventional techniques.

The neutralizing antibodies which react specifically with the compositions of the present invention containing a perturbed oligosaccharide moiety can be formulated to confer short-term passive immunity to the host. Adjuvants are not needed in this type of preparation because the object is not to stimulate an immune response, but rather to inactivate or bind a viral pathogen. Thus, any suitable pharmaceutical carrier can be used. Passive immunization using such preparations can be utilized on an emergency basis for immediate protection of unimmunized individuals exposed to special risks of viral infections. Additionally, such preparations can be used prophylatically for viral infections such as measles and hepatitis.

In its most general form, the method of this embodiment of the invention encompasses a method for protection of an animal or a human from an infection induced by a virus, comprising: administering to an animal or human an effective amount of a vaccine formulation which comprises a virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety.

Such formulations can be administered to a host by routes including but not limited to: intradermal, intramuscular, intraperitoneal, intravenous and subcutaneous.

4.3. Viruses and Viral Antigens

The viruses, viral antigens and fragments thereof which are intended to be encompassed by the present invention include a wide variety of viruses such as DNA and RNA viruses including but not limited to retroviruses.

Specific examples include: DNA viruses such as: Adenoviridae such as adenoviruses subgroups B, C, D, E, F and G, etc; Herpesviridae such as herpes simplex I and II, cytomegalovirus, Epstein-Barr virus, varicella-zoster, etc.; Orthomyxoviridae such as influenza viruses, etc; Hepadnaviridae such as hepatitis B, hepatitis non-A, non-B, etc.; Parvoviridae such as parvoviruses, etc.; RNA viruses such as Togaviridae such as rubella, etc.; Paramyxoviridae such as measeles, parainfluenza, respitatory syncytial virus, etc.; Flaviviridae such as dengue virus types 1–4, yellow fever virus, tick-borne fever viruses, etc.; Rhabdoviridae such as rabies, vesicular stomatitis virus, Marburg-Ebola virus, etc.; Bunyaviridae such as Rift Valley Fever, California encephalitis virus group, sand fly fever virus, etc.; Arenaviridae such as Lassa fever virus, Junin virus, lymphocytic choriomeningitis virus, etc.; Reoviridae such as rotovirus, etc.; Picornaviridae such as polio virus, coxsackieviruses, hepatitis A virus, rhinovirus, etc.; Retroviridae such as human lymphoadenopathy-associated virus (LAV, HIV, HTLV-III), human T-cell lymphotrophic virus types I, II, III, feline leukemia virus, etc.

The following Examples are given for the purpose of illustration and not by way of limitation on the scope of the invention.

In the ELISA assays described below in which the virus was covalently attached to the microtiter plate, the microtiter plates were pre-saturated using either 200 ul of 0.5% calf IgG in 0.14 M NaCl or 300 ul of 0.5% bovine serum albumin in 0.5 M Tris-citrate buffer containing 0.1% Tween-20, pH 8.1 in order to eliminate the non-specific adsorption of virus or serum proteins or IgG. In the ELISA assays described below in which the virus was attached to the plate via adsorption, the virus was allowed to adsorb to the plate and then the plates were saturated using either calf IgG in 0.14 M NaCl or bovine serum albumin in 0.5 M Tris-citrate buffer.

5. EXAMPLES: DETERMINATION OF NEUTRALIZING ANTIBODIES IN PURIFIED HUMAN IgG FROM SERA

5.1. Detection of Neutralizing Anti-CMV Antibodies in Human IgG

The following series of experiments demonstrate that ELISA assays in which the oligosaccharide moiety of the viral antigen was perturbed according to the present invention are useful for determining the titer of virus-neutralizing antibodies. In contrast, conventional ELISA assays in which the virus was either adsorbed or covalently attached without perturbation of the oligosaccharide moiety are not.

The antigen used in the assays was whole cytomegalovirus (CMV) obtained from homogenates of cultured human fibroblast cells (MRC_5) infected with CMV for six to eight days. The microtiter wells containing an equivalent amount (about 1 ug/well) of CMV were prepared for the various ELISA assays as follows:

(A) ELISA'S Using Antigen With Perturbed Oligosaccharide.

(1) Virus Covalently Attached Via Oxidized Oligosaccharide Moiety:

The carbohydrate moiety of CMV was oxidized using sodium periodate ($NaIO_4$) as described in Section 4 and covalently coupled to a reactive amine on a side chain of the polyhydrazidostyrene of the well. Addition of a reactive amine group to polystyrene (or the microtiter well) was carried out by the method of Chin and Lanks [Anal. Biochem, 83: 709–19 (1977)]. Polystrene was first converted to nitrostyrene which was then reduced to aminostyrene. Polyaminostyrene was then converted to polyhydrazidostyrene in a two-step process: (1) polyaminostyrene was succinylated; and then (2) an amide bond was formed betwen hydrazine and

the succinylated polyaminostyrene using carbodiimide. (Ox Oligosaccharide Attached ELISA).

(2) Virus Covalently Attached Via Amine Group, Oxidized Oligosaccharide Moiety:

The CMV virus was covalently attached to the polycarbostyrene via a peptide bond formed using carbodiimide to couple an amine residue of the virus to an activated carboxyl of the polycarbostyrene. The carbohydrate moiety of the virus was then oxidized in situ using $NaIO_4$ as described in Section 4. (Amine Attached Oligosaccharide Ox ELISA).

(B) Conventional ELISA'S Using Antigen with Non-Perturbed Oligosaccharide.

(1) Virus Covalently Attached Via Amine Group:

CMV was attached using carbodiimide to form a peptide bond between an amine group of an amino acid residue of the virus and a free carboxyl group of a polycarbostyrene of the well. (Amine Attached ELISA).

(2) Virus Non-Covalently Attached:

CMV was simply non-covalently fixed by adsorption onto a non-modified polystyrene well. (Adsorption ELISA).

Partially purified human IgG was obtained from human serum samples using conventional ammonium sulfate precipitation techniques. After precipitation, the tubes were centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded, the pellet was washed, recentrifuged and resuspended in 0.14 M NaCl. The partially purified human IgG samples were serially diluted in Buffer I of the following composition:

Calf IgG	0.5%
NaCl	0.14 M
Glycine	0.1 M
Sodium borate	0.05 M
Synperonic PE/L62	0.10% (v/v),

adjusted to pH 8.1 with 1.0 M HCl. 100 ul of each dilution was distributed in the wells of the plate containing the CMV. After two hours incubation at 37° C., the plates were washed in the Buffer I, but without calf IgG.

Then 100 ul of a goat serum solution containing anti-human IgG labeled with alkaline phosphatase, diluted to 1/1000 in the following Buffer II, was introduced into each well:

Bovine serum albumin	1.0%
NaCl	0.14 M
Glycine	0.1 M
Borate	0.05 M
Synperonic PE/L62	0.10% (v/v)

After a contact time of one hour at 37° C., the wells were washed with Buffer II but without bovine serum albumin (BSA). The enzymatic activity was determined at 37° C. using p-nitrophenyl phosphate as substrate at a concentration of 0.2% (p/v) dissolved in a buffer containing: 2-amino-2-methyl-1-propanol (0.625 M) and $MgCl_2$ (2.0 mM), pH 10.25. The optical density was measured at 405 nm either every five minutes during a period of 30 minutes or after 30 minutes of incubation.

A conventional CMV infective power neutralization assay was performed using MRC_5 cells in culture as described by Krech et al., Z. Immun.-Forsch, Bd. 141S: 411–29 (1971).

Results are illustrated in Table 1. The antibody titer obtained using the ELISA assays and the conventional virus neutralization assays were calculated as a protein concentration of 1 mg/ml of non-diluted IgG sample. Table 2 presents the linear correlation coefficients obtained when the titers obtained by the various assays were compared.

TABLE 1

TITERS OF CMV-NEUTRALIZING ANTIBODIES IN PURIFIED IgG

IgG Sample	Neutralization Titer	Ox Oligosaccharide Attached ELISA Titer	Amine Attached Oligosaccharide Ox ELISA Titer	Amine Attached ELISA Titer	Adsorption ELISA Titer
1	128	50	<50	400	67
2	256	50	<50	200	144
3	256	100	100	400	519
4	512	400	100	800	1087
5	64	<50	<50	50	54
6	128	50	50	200	67
7	256	200	200	800	432
8	465	364	181	727	2780
9	621	485	485	485	1005
10	512	400	400	>1600	2923
11	128	200	100	400	387
12	256	100	100	1600	593
13	64	<50	50	54	100
14	256	50	50	455	400

TABLE 2

COMPARISONS OF ASSAYS FOR CMV-NEUTRALIZING ANTIBODIES

Assay	Linear Correlation Coefficients				
Neutralization	1				
Adsorption ELISA	0.74	1			
Ox Oligosaccharide Attached ELISA	0.90	0.75	1		
Amine Attached ELISA	0.52	0.62	0.41	1	
Amine Attached Oligosaccharide Ox ELISA[a]	0.78 (0.87)	0.65 (0.64)	0.82 (0.93)	0.46 (0.48)	1
	Neutralization	Adsorption ELISA Attached	Ox Oligosaccharide ELISA	Amine Attached ELISA	Amine Attached Oligosaccharide

TABLE 2-continued

COMPARISONS OF ASSAYS FOR CMV-NEUTRALIZING ANTIBODIES	
Assay	Linear Correlation Coefficients
	Ox ELISA

[a]The number in parentheses represents the correlation coefficient obtained when one aberrant titer value was discarded.

As demonstrated in Tables 1 and 2, the titer obtained using ELISA assays according to the present invention, i.e., the Ox Oligosaccharide Attached ELISA and the Amine Attached oligosaccharide Ox ELISA was highly positively correlated with the titer obtained using the conventional neutralization Assay (correlation coefficients, respectively: 90 and 0.87). On the other hand, the titer obtained using the conventional Amine Attached ELISA showed no significant correlation with the titer of neutralizing antibody (correlation coefficient: 0.52). The Amine Attached ELISA showed much weaker, non-significant correlation with titer of neutralizing antibody (correlation coefficient: 0.74).

Table 2 demonstrates further that there was a significant positive correlation between the titer obtained using the Ox Oligosaccharide Attached ELISA and the Amine Attached Oligosaccharide Ox ELISA (correlation coefficient: 0.93). At the same time, however, there was no significant correlation observed between the titers obtained using the Adsorption ELISA and the Amine Attached Oligosaccharide Ox ELISA, or the Amine Attached Oligosaccharide Ox ELISA and the Amine Attached ELISA. This indicates that the significant correlation observed between the titers of neutralizing antibody using the Neutralization Assay and both the Amine Attached Oligosaccharide Ox ELISA and the Ox Oligosaccharide Attached ELISA is not related to the method of covalent attachment, but rather may be related to the perturbation of the oligosaccharide moiety achieved by oxidation. Moreover, these results suggest further that it does not matter whether the oligosaccharide perturbation occurs prior to or following covalent attachment of the virus to the microtiter well.

5.2. Reproducibility of Neutralizing Antibody Titer

The following experiment demonstrates the reproducibility of results obtained using an ELISA assay in which the virus was covalently attached to a insoluble support via an oxidized carbohydrate moiety of the virus.

A series of ELISA assays to determine the titer of neutralizing anti-CMV antibodies was performed as described in Section 5.1 in which CMV was covalently attached to a reactive amine on a side chain of an insoluble support via an oxidized carbohydrate moiety of the CMV antigen. The samples used were purified IgG obtained from the same serum samples used for the experiments described in Section 5.1. One set of ELISA's were performed on one aliquot of purified IgG's, and a duplicate set of assays were performed on another aliquot of the same IgG's some 17½ months later. The samples were stored frozen at −70° C. during the interim. Results are presented in Table 3.

TABLE 3

REPRODUCIBILITY OF CMV NEUTRALIZING ANTIBODY ASSAY		
Sample No.	Antibody Titer - ELISA Virus Covalently Attached Via Oxidized Oligosaccharide	
	Experiment 1	Experiment 2
1	50	40
2	50	40
3	100	160
4	400	320
5	<50	160
6	50	40
7	200	160
8	364	290
9	485	388
10	400	640
11	200	160
12	100	80
13	<50	80
14	50	80

As demonstrated in Table 3, the results of antibody titers obtained using the ELISA assay in which the virus was covalently attached via a perturbed oligosaccharide moiety are highly reproducible.

6. EXAMPLE: DETECTION OF NEUTRALIZING ANTIBODIES IN SERUM SAMPLES

The following series of assays demonstrate that an ELISA assay in which the oligosaocharide moiety of a virus antigen was perturbed is useful for determining the titer of neutralizing antibody in human serum samples.

An ELISA assay was performed as described in Section 5.1 in which the oligosaccharide moiety of the CMV virus was oxidized and covalently coupled to a hydrazido group on the polyhydrazidostyrene of the microtiter well. A conventional ELISA assay was performed as described in Section 5.1 in which the CMV was merely adsorbed to the microtiter well. A virus neutralization assay as described in Section 5.1 was also performed.

Results of all three assays are compared in Table 4.

TABLE 4

Serum Sample No.	Adsorption ELISA Titer[a]	Neutralization Assay Titer	Oxidized Oligosaccharide Attached ELISA Titer[b]
1	6400	160	125
2	26600	320	250
3	102400	640	2000
4	25600	640	1000
5	102400	2560	4000
6	25600	2560	4000

[a]Correlation coefficient between Neutralization Assay Titer and Adsorption ELISA Titer: +0.37.
[b]Correlation coefficient between Neutralization Assay Titer and Oxidized Oligosaccharide Attached ELISA Titer: +0.96.

As demonstrated in Table 4, there was a highly significant positive correlation between the titer of antibodies in human serum samples measured by the Neutralization Assay and by an ELISA assay in which the oligo-

saccharide moiety of CMV was oxidized and covalently coupled to the microtiter well. Thus using polyclonal sera, this ELISA assay is "predictive" of the immunocompetent status of the patient. In contrast, no correlation was observed between the antibody titer measured by the Neutralization Assay and that obtained using a conventional ELISA in which non-perturbed CMV virus was merely adsorbed to the microtiter well

7. EXAMPLE: PERTURBATION OF OLIGOSACCHARIDE MOIETY OF VIRUS AND ATTACHMENT OF VIRUS TO A SOLID SUPPORT

As suggested by results presented in Section 5.1 above, when a perturbed antigen is used to determine the titer of neutralizing antibodies in an ELISA format in which the antigen is covalently attached to the microtiter well, it does not matter whether the oligosaccharide moiety is perturbed before or after covalent attachment to the microtiter well. The following series of experiments was performed to investigate the effect of perturbation of the oligosaccharide moiety according to the present invention upon the ability of the virus to attach to a microtiter plate.

The oligosaccharide moiety of CMV virus was perturbed by oxidization using $NaIO_4$ for 16 hours at 4° C. as described in Section 4. ELISA assays for anti-CMV antibodies were conducted as described in Section 5.1, in which: (1) CMV having a perturbed oligosaccharide moiety in PBS was adsorbed to a polystryene microtiter plate. (2) CMV having a perturbed oligosaccharide moiety was covalently coupled via the carbohydrate moiety to a reactive amine group of a polyhydrazidostyrene microtiter plate in the presence of phosphate buffer containing 0.1% Tween-20 (PBT). PBT was used to prevent non-covalent adsorption of CMV to the polyhydrazidostyrene plate. (3) Non-perturbed CMV in PBS was adsorbed to a polystyrene microtiter plate.

Results are illustrated in Table 5.

TABLE 5

COMPARISON OF ELISA TITERS OF CMV WITH PERTURBED AND NON-PERTURBED OLIGOSACCHARIDE

IgG No.	Ox CMV Adsorbed in PBS[a]	Ox CMV Covalently in PBT[b]	Non-Ox CMV Adsorbed in PBS[a]
Y 287	4000	4000	8000
Y1096	1000	1000	8000

[a]CMV either native or having a perturbed oligosaccharide moiety in PBS (phosphate buffer saline, pH 7.4) was immobilized by adsorption onto polystyrene microtiter plates.

[b]CMV having a perturbed oligosaccharide moiety in PBT (50 mM phosphate buffer, 0.1% Tween-20, pH 6.0) was covalently coupled to polyhydrazidostyrene of microtiter plates.

As demonstrated in Table 5, there was no difference in ELISA titers obtained in which a perturbed antigen was either covalently attached or merely adsorbed to the micro-titer well. When the perturbed CMV was incubated in the microtiter wells in the presence of PBT, the titer obtained was zero because the perturbed virus does not adsorb in the presence of Tween-20 (results not shown).

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

What is claimed is:

1. A method for detecting virus neutralizing antibodies in an aqueous sample, comprising:

(a) contacting (i) a ligand which comprises a virus, viral antigen or fragment thereof, said virus, viral antigen or fragment thereof having perturbed oligosaccharide moiety, in which said perturbed oligosaccharide moiety was obtained by oxidation, by means of an agent selected from the group consisting of periodic acid, salts thereof, paraperiodic acid, salts thereof, metaperiodic acid, salts thereof, and oxidase enzymes, of an unperturbed oligosaccharide moiety, with (ii) an aqueous sample suspected of containing virus neutralizing antibodies in an assay system selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, an agglutination assay, and an immunofluorescence assay; and

(b) detecting any reaction with the ligand; in which any reaction with the ligand indicates the presence of neutralizing antibodies in the sample.

2. A method for detecting and quantitating virus neutralizing antibodies in an aqueous sample, comprising:

(a) contacting (i) a ligand which comprises a virus, viral antigen or fragment thereof, said virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety, in which said perturbed oligosaccharide moiety was obtained by oxidation, by means of an agent selected from the group consisting of periodic acid, salts thereof, paraperiodic acid, salts thereof, metaperiodic acid, salts thereof, and oxidase enzymes, of an unperturbed oligosaccharide moiety, with (ii) an aqueous sample suspected of containing virus neutralizing antibodies in an assay system selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, an agglutination assay, and an immunofluorescence assay;

(b) detecting any reaction with the ligand; in which any reaction with the ligand indicates the presence of neutralizing antibodies in the sample; and

(c) comparing any reaction of ligand and antibodies to that of a standard.

3. The method according to claim 1 or 2, in which the sample is an aliquot of a body fluid.

4. The method according to claim 3, in which the body fluid is serum or plasma.

5. The method according to claim 1 or 2, in which the aqueous sample is an aliquot of a solution of a monoclonal antibody.

6. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize a virus selected from the group consisting of: adenoviridae, herpesviridae, orthomyxoviridae, hepadnaviridae, parvoviridae, togaviridae, paramyxoviridae, flaviviridae, rhabdoviridae, bunyviridae, reoviridae, picornaviridiae and retroviridae.

7. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize cytomegalovirus.

EXHIBIT A

8. The method according to claim 1 or 2 in which the virus neutralizing antibodies neutralize herpes simplex I or herpes simplex II virus.

9. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize hepatitis virus.

10. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize rubella virus.

11. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize measles virus.

12. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize parainfluenza virus.

13. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize dengue virus.

14. The method according to claim 1 or 2, in which the virus neutralizing antibodies neutralize human lymphadenopathy-associated virus (LAV, HTLV-III, HIV).

15. The method according to clain: 1 or 2, further comprising the step of dissociating any immune complexes which may be present in the aqueous sample prior to contacting the ligand with the sample.

16. The method according to claim 1 or 2, in which the aqueous sample contains artially purified immunoglobulin.

17. A method for detecting virus neutralizing antibodies in an aqueous sample, comprising:

(a) contacting a virus, viral antigen or fragment thereof with oxidizing agent selected from the group consisting of periodic acid, salts thereof, paraperiodic acid, salts thereof, metaperiodic acid, salts thereof, and oxidase enzymes to form a ligand which comprises a virus, viral antigen or fragment thereof, said virus, viral antigen or fragment thereof having a perturbed oligosaccharide moiety;

(b) contacting the ligand with an aqueous sample suspected of containing virus neutralizing antibodies in an assay system selected from the group consisting of an enzyme-linked immunosorbent assay, a radioimmunoassay, an agglutination assay, and an immunofluorescence assay;

(c) detecting any reaction with the ligand; in which any reaction with the ligand indicates the presence of neutralizing antibodies in the sample.

18. The method according to claim 17, which further comprises:

(d) comparing any reaction of the ligand and antibodies to that of a standard in order to quantitate the virus neutralizing antibodies.

* * * * *

EXHIBIT A

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 4,853,326

DATED : August 1, 1989

INVENTOR(S) : Gerard A. Quash, et. al.

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

On the Title page, item [73], Assginee: add Institut National de la Sante et de la Recherche Medicale Paris, France.

Signed and Sealed this

Twenty-eighth Day of June, 1994

Bruce Lehman

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trodemarks

[redacted]

EXHIBIT A

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 4,853,326

DATED : August 1, 1989

INVENTOR(S) : Gerard A. Quash, John D. Rodwell, Thomas J. McKearn and Jean Pierre Ripoll

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title Page:

[73] Add to Assignee:
Institut National de la Sante et de la Recherche Medicale
Paris, France.

Signed and Sealed this

Thirteenth Day of September, 1994

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

EXHIBIT B

US 20070202099A1

(19) **United States**

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(54) **ANTIBODY DRUG**

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(21) Appl. No.: **10/594,773**

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§ 371(c)(1),
(2), (4) Date: **Sep. 29, 2006**

(30) **Foreign Application Priority Data**

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Publication Classification

(51) **Int. Cl.**
A61K 39/395 (2006.01)

(52) **U.S. Cl.** **424/143.1**; 514/15; 514/12

(57) **ABSTRACT**

The present invention provides an agent for improving the blood stability of an endogenons ligand, which comprises an antibody that has affinity to the mammalian endogenons ligand bnt snbstantially does not neutralize the same, and the above-described agent for the prophylaxis and/or treatment for a disease for which an increase in the blood concentration of the endogenous ligand and/or an prolonged blood half-life is prophylactically or thcrapcntically cffcctive. Provided that the above-described agent is administered alone to a mammal withont co-administering the same or substantially the same compound as the endogenous ligand, the blood stability of the endogenous ligand increases and the receptor activity-regulatory action thereof is enhanced.

EXHIBIT B

FIG. 1

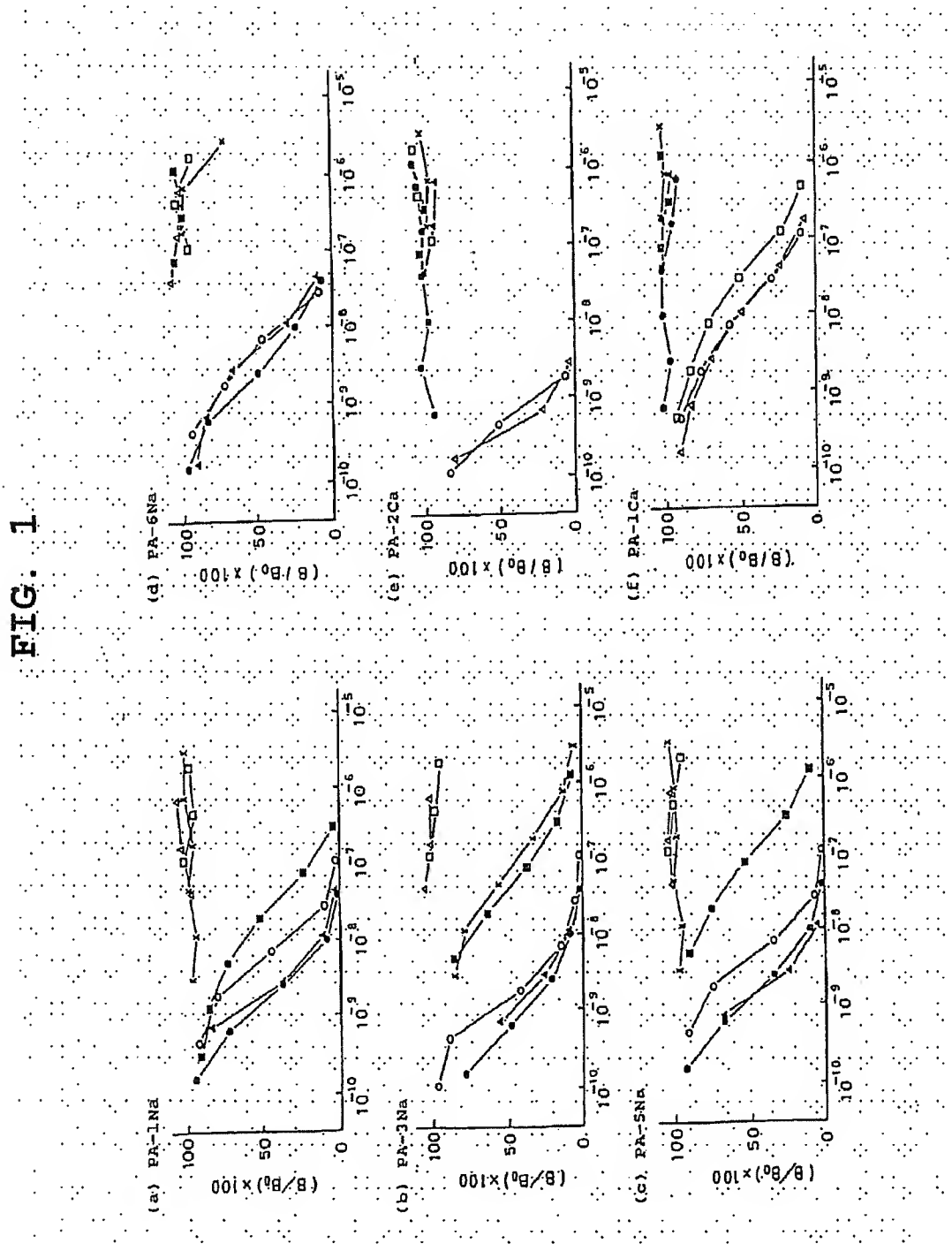


EXHIBIT B

FIG. 2

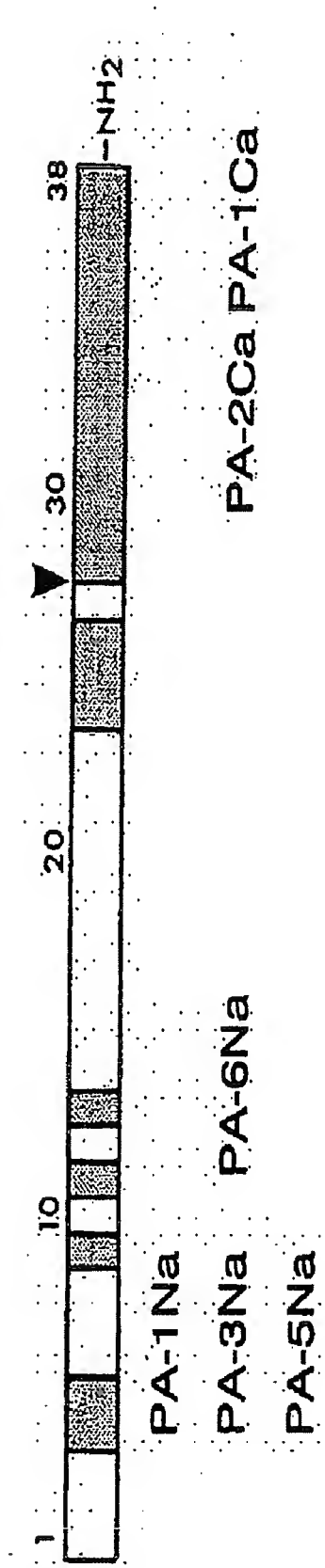


EXHIBIT B

FIG. 3

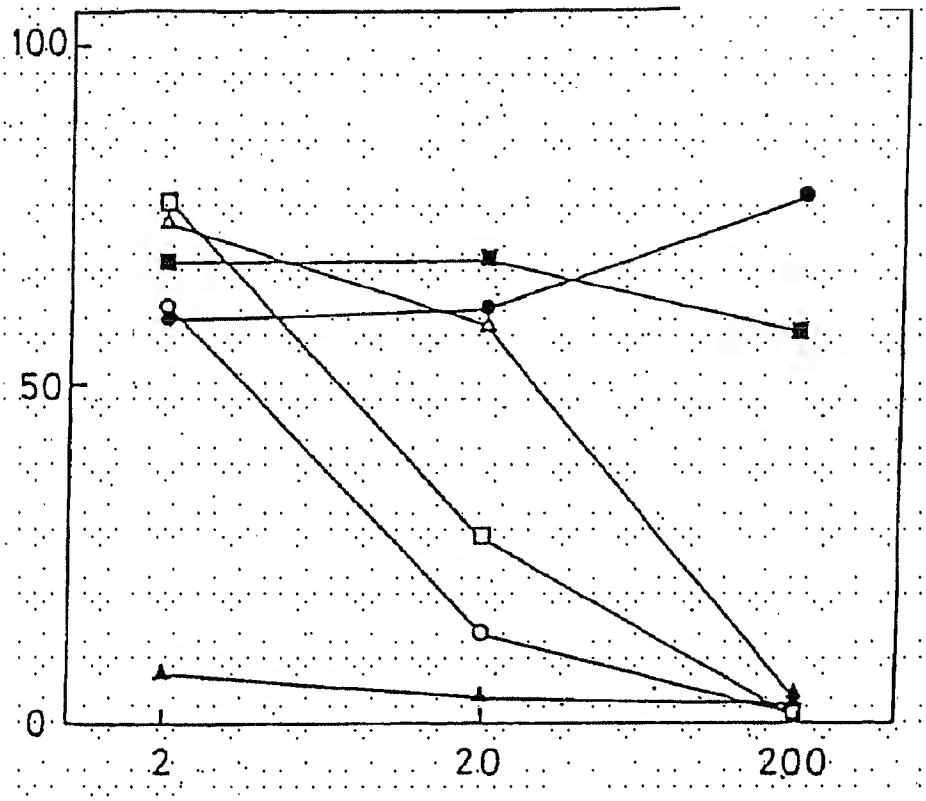


EXHIBIT B

FIG. 4

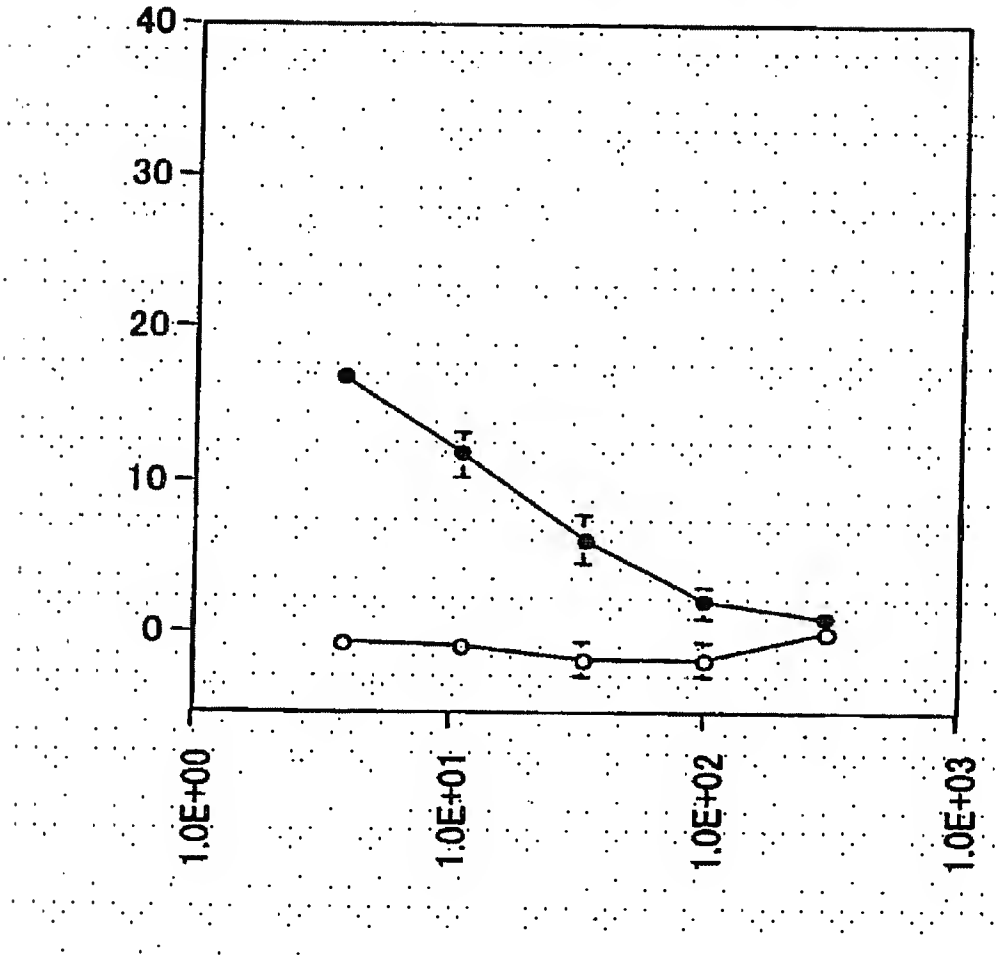


EXHIBIT B

FIG. 5

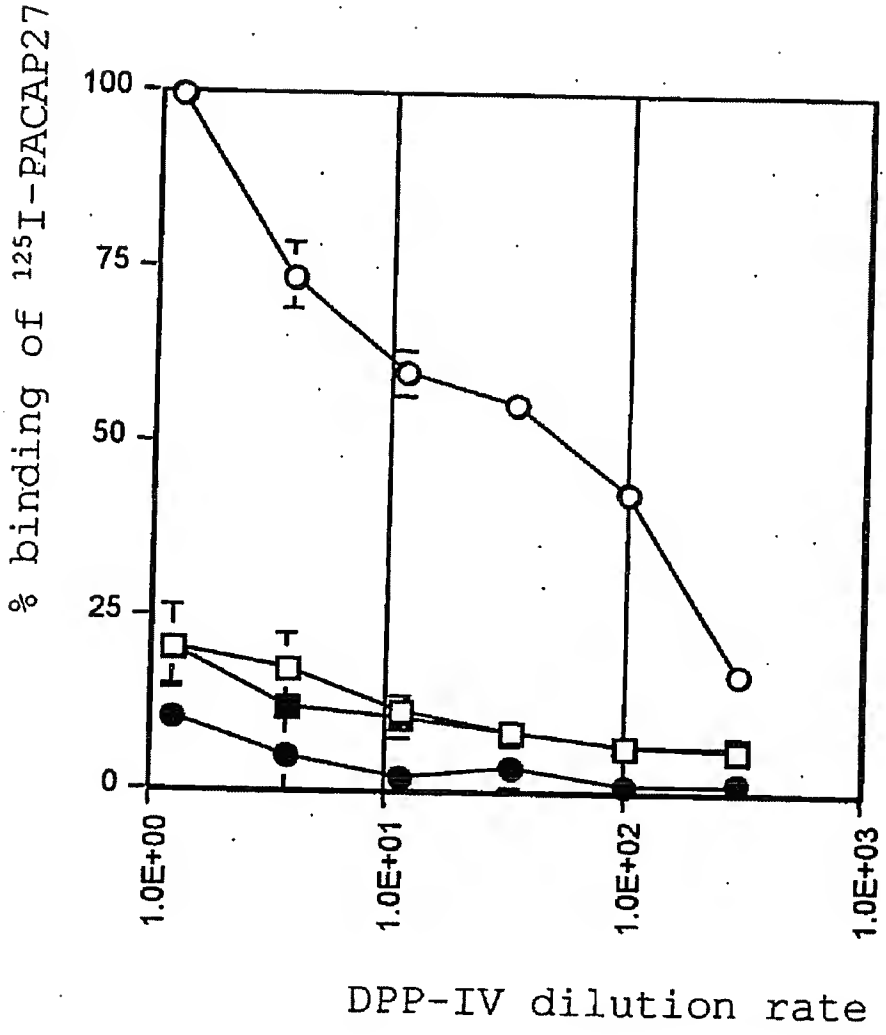


EXHIBIT B

FIG. 6A

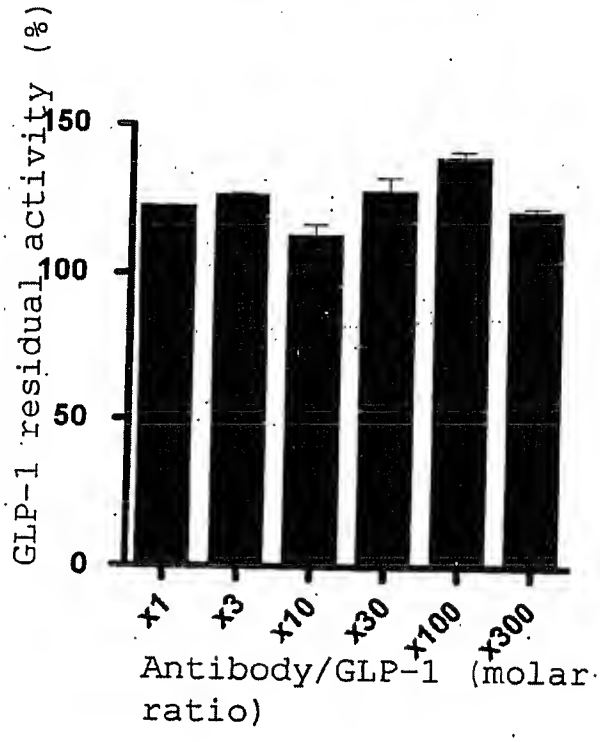


FIG. 6B

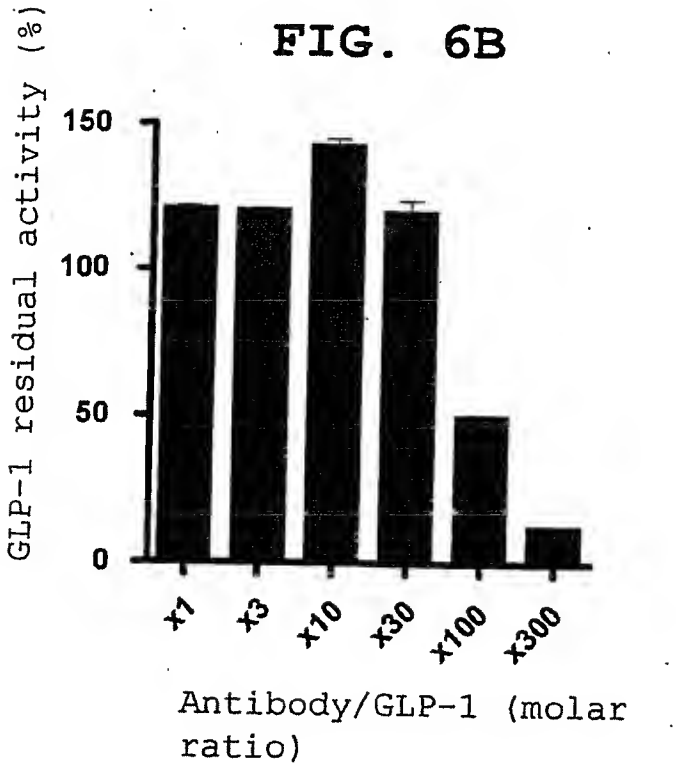


EXHIBIT B

FIG. 7

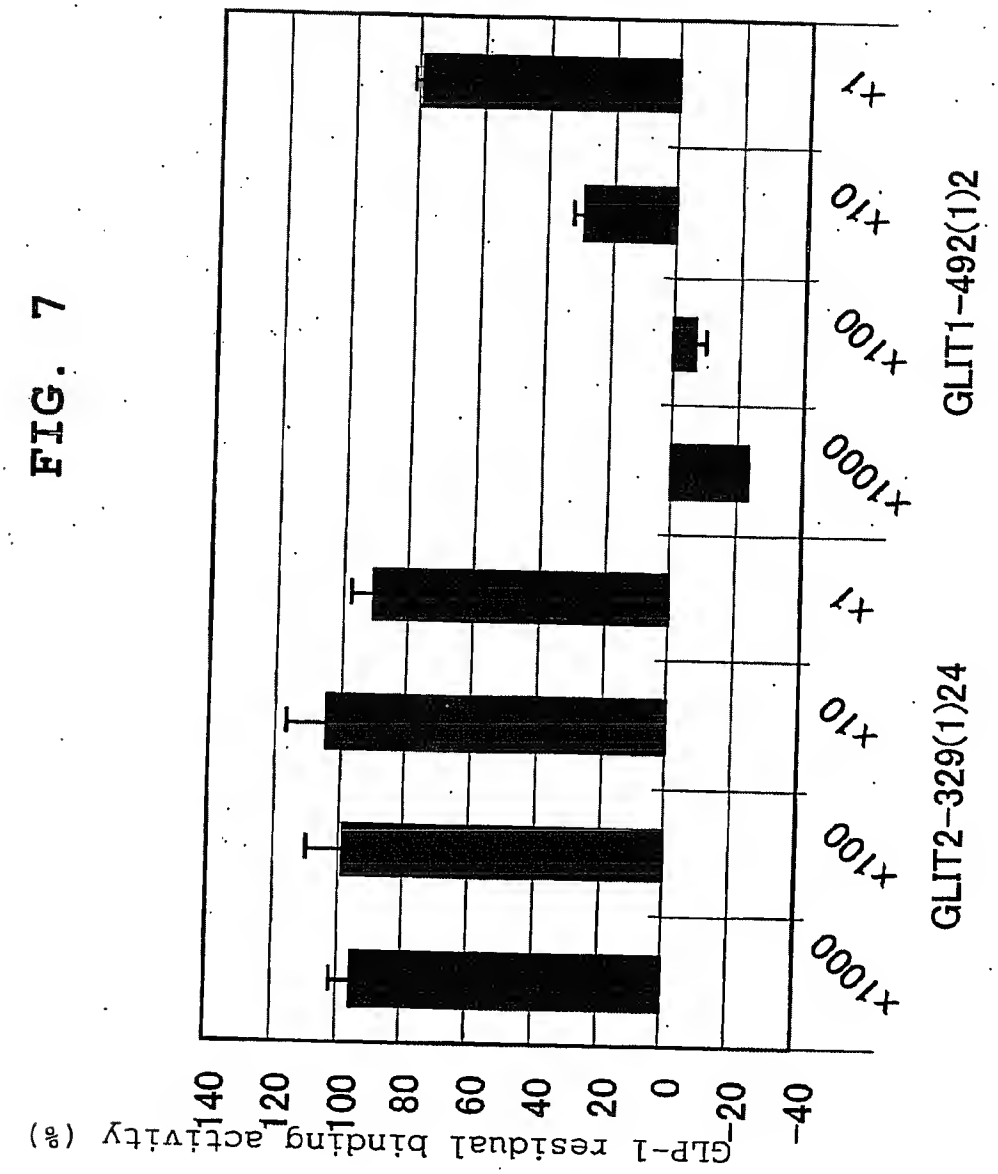


EXHIBIT B

FIG. 8

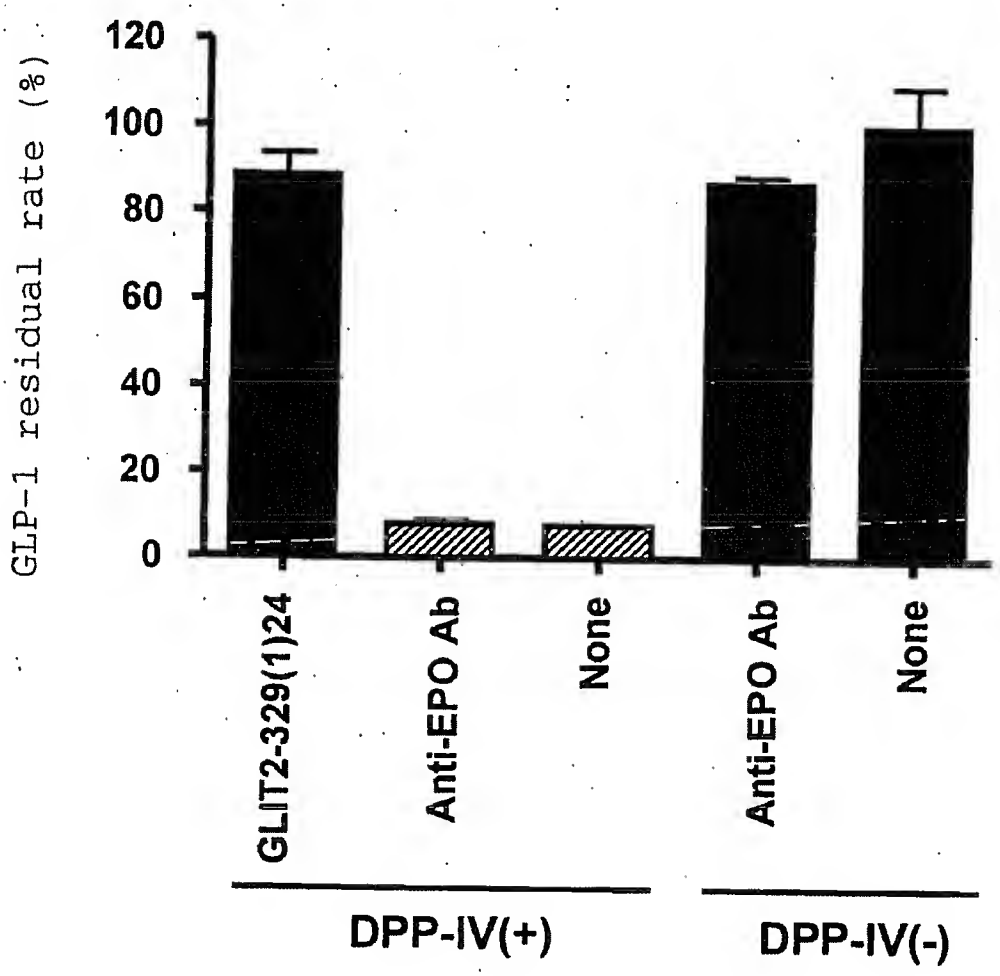
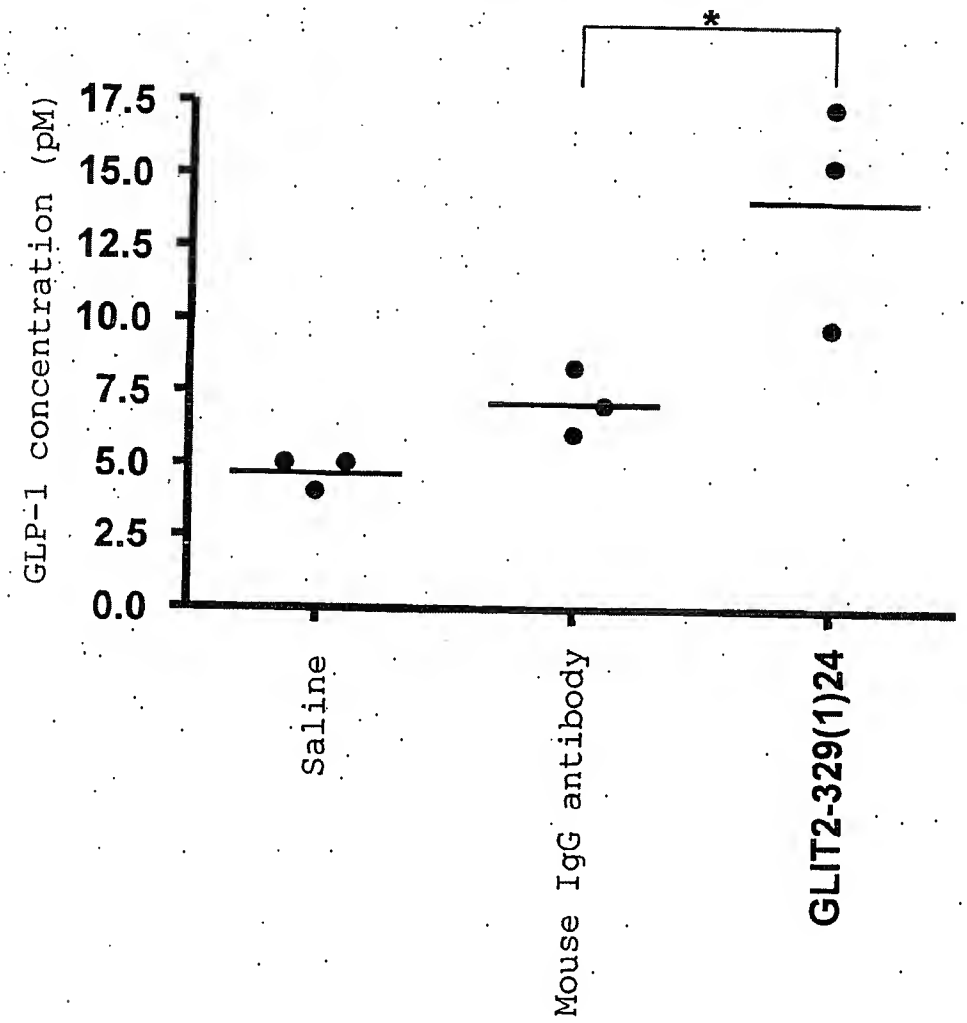


EXHIBIT B

FIG. 9



* : P<0.05

EXHIBIT B

ANTIBODY DRUG

TECHNICAL FIELD

[0001] The present invention relates to a novel pharmaceutical use of an antibody. More specifically, the present invention relates to a use of an antibody that has affinity for an endogenous ligand but does not completely neutralize the same, for enhancing the receptor activation action of the ligand by improving the stability of the ligand in blood.

BACKGROUND ART

[0002] Antibodies have excellent properties for pharmaceutical products such as low incidence of side effects and long sustainability of efficacy because of their high specificity and long half-life in blood. The first antibody medicine in the modern sense that targets a disease-specific antigen was a mouse anti-CD3 monoclonal antibody approved as a therapeutic agent for acute rejection in the United States in 1986 (trade name: Orthoclone); since then, however, development of antibody medicines had long been at a standstill because mouse monocloual antibodies have drawbacks, for example, when they are administered to humans, anti-mouse immunoglobulin (Ig) human antibodies known as HAMA (Human Anti-Mouse Antibodies), are produced and cause efficacy reductions due to the shortened half-life of the drug and anaphylactic symptoms, and when antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) is utilized, the action remarkably decreases in the human body in the case of mouse Fc.

[0003] However, in the 1990s, as production technologies for chimeric antibodies, humanized antibodies, and fully human antibodies were established and the problem of immunogenicity and efficacy reductions was resolved, antibody medicines became highlighted again, and therapeutic drugs mainly for intractable diseases such as cancers, graft rejection, cardiovascular diseases, infectious diseases, and autoimmune diseases were brought into actual application one by one; more than 100 antibody medicines, including those in the clinical stage, have been developed to date.

[0004] Most of the conventional antibody medicines, including those under development, are based on any of the modes of action: (1) binding to the target antigen and inhibiting (namely, neutralizing) the function thereof [e.g., anti-TNFα antibody (trade name: Remicade), anti-RSV surface protein antibody (trade name: Synagis)]; (2) functioning as a carrier for delivering a drug (for example, chemotherapeutic agent, toxin, radioisotope, cytokine, etc.) to pathogenic cells that express the target antigen [e.g., ^{90}Y-bound anti-CD20 antibody (trade name: Zevalin), calicheamycin-bound anti-CD33 antibody (trade name: Mylotarg)]; and (3) attacking the cells that express the target antigen by utilizing ADCC or CDC [e.g., anti-HER2 antibody (trade name: Herceptin), anti-CD20 antibody (trade name: Rituxan)].

[0005] By contrast, as a new generation of antibody medicines, development of what are called agonist antibodies, which are intended to enhance the function of the target antigen, rather than neutralizing the target antigen or killing the antigen-expressing cells, is being considered. For example, there has been proposed a method comprising cross-linking monomer molecules of a receptor that forms an oligomer by its ligand binding to activate signal transduction, like a tyrosine kinase type receptor, by using an antibody against such monomer molecules to activate the receptor (see, for example, WO 01/79494).

[0006] However, with regard to antibody medicines that target humoral biologically active peptides (proteins) serving as ligand molecules for receptors on the cell surface, such as cytokines and growth factors, neutralizing antibodies that inhibit the function of the target antigen (i.e., receptor activation action) are mainly utilized.

[0007] Although biologically active peptides (proteins) are utilized as pharmaceuticals since they are ligand molecules that exhibit various pharmacological actions in a living organism, these are highly susceptible to peptidases (proteases) and are likely to undergo renal clearance because of their small molecular weights so that their active forms generally have short half-life in the living organism. For this reason, the biological active peptides (proteins) have a drawback in terms of efficacy sustainability and a frequent dosing is often necessary. Although there have been attempts to extend the blood half-life of a biologically active protein such as a cytokine to improve the efficacy sustainability by using in combination an antibody that binds to the protein at a site where the protein's biological activity is not affected, in administering a pharmaceutical with the protein as an active ingredient (see, for example, Japanese Patent Kohyo publication No. SHO-60-502104), no such approaches have been in actual application.

[0008] Also, unlike other biotechnology-based pharmaceutical products such as cytokines, which exhibit their efficacy at low doses, clinical doses of conventional antibody medicines are generally enormous, in some cases, are as much as several hundred milligrams. Currently, antibody medicines are produced as recombinants using animal cells such as CHO cells, and are very expensive due to limitations on the amount of antibody produced and culture scale; therefore, the indications of antibody medicines are now limited to intractable diseases for which no adequate therapeutic effect is obtained with any existing therapeutic drug. Although antibody medicines under clinical development include therapeutic drugs for chronic diseases affecting many patients (e.g., rheumatoid arthritis), it is critical to overcome the cost-related problems before the antibody medicine market is expanded to cover the area of lifestyle-related diseases such as diabetes and hyperlipidemia.

[0009] Furthermore, it is also important from the viewpoint of safety to reduce the dose of an antibody medicine. The majority of antibody medicines under development are either chimeric or humanized antibodies, however, they sometimes cause production of an anti-Ig human antibody (HACA or HAHA) because these comprise a mouse-derived variable region or CDR. Although the problem of anti-Ig human antibody induction is nearly completely resolved by establishing a technology for producing a fully human antibody, the possibility of production of anti-human Ig human antibody with administration of an antibody in large amounts cannot be ruled out because any antibody has the property of being non-self to the host as the intrinsic fate thereof.

DISCLOSURE OF THE INVENTION

[0010] It is an object of the present invention to provide a new generation of antibody medicine based on a different

basic concept from that of the conveutional antibody medicines. Particularly, the present invention is directed to providing a novel antibody medicine that is useful as a therapeutic drug iu the area of diseases for which existing antibody medicines do not have a sufficient therapeutic effect and the market scale is large, such as life style-related diseases. In another aspect, the present invention is directed to reducing the amount of antibody used per course of treatment by providiug an antibody uedicine capable of having a therapeutic effect at much lower doses compared with couveutioual antibody medicines, to thereby contribute to medical economics.

[0011] The present inventiou is partially based on the fiuding that an anti-PACAP monoclonal antibody recognizing a certain partial sequence of pituitary adenylate cyclase-activating polypeptide (PACAP) as the antigenic determinant does uot inhibit the action of PACAP [adenylate cyclase (AC) activation action], and that when the anti-PACAP antibody is administered alone to a mouse, endogenous PACAP which is uormally uot detected in blood, is detected as a complex with the autibody (i.e., stabilized in blood). Based on this finding, the preseut iuventors thought that by admiuistering an antibody that has affinity for an endogcnous ligand of the recipicnt animal and does uot completely inhibit the action of the ligand (non-neutralizing antibody) alone to the animal (i.e., without administering the ligand or an equivalent thereof), it would be possible to improve the blood stability thereof (i.e., increase the ligand concentration in blood) while retaining the physiological action of the endogenous ligand, and, as a result, to enhance the receptor activation action of the ligand. Hence, the present inventors newly prepared a nou-ueutralizing antibody against glucagon-like peptide-1 (GLP-1), succeeded in raising the plasma GLP-1 concentration to a level showing efficacy by administering this to a rat, aud developed the present invention.

[0012] Accordiugly, the present invention relates to

[1] an ageut for improving the blood stability of a mammalian endogenous ligand, which comprises an antibody that has an affinity to the endogenous ligand but does not neutralize the same substantially,

[2] the agent of [1] above, wherein the improved blood stability of the eudogenous ligand results in the enhancement of receptor activity-regulatory action thereof,

[3] the ageut of [1] above, wherein the neutralizing activity of the antibody is about 80% or less,

[4] the agent of [1] above, wherein the blood conceutration of the eudogenous ligand becomes about twice or more compared to the case where the antibody is not administered,

[5] the agent of [1] above, wherein the blood half-life of the complex of the endogenous ligaud and the antibody is about twice or more as that of the endogenous ligaud alone,

[6] the agent of [1] above, wherein the blood half-life of the free endogenous ligand is about one week or less,

[7] the agent of [1] above, wherein the endogenous ligand is a peptidic compound,

[8] the agent of [7] above, wherein the endogenous ligand is one agaiust a G proteiu-coupled receptor,

[9] the agent of [8] above, wherein the endogenous ligand is one belonging to secretin/glucagon super family,

[10] the agent of [9] above, wherein the endogenous ligand is selected from the group cousisting of GLP-1, calcitonin, PACAP, VIP and analogs thereof,

[11] the ageut of [8] above, wherein the endogenous ligand is selected from the group cousistiug of LHRH, metastin, GPR7/GPR8 ligand, MSH, ghrelin, apelin and analogs thereof,

[12] the agent of [7] above, wherein the eudogenous ligand is selected from the group consisting of EPO, TPO, insulin, interferon, growth hormone, GM-CSF, leptiu, adipouectin and analogs thereof,

[13] the agent of [7] above, wherein the endogenous ligand is selected from the group consistiug of ANP, BNP, CNP, betacellulin, betacellulin-64, adreuomedullin and aualogs thereof,

[0013] [14] the ageut of [1] above, which is for the prophylaxis and/or treatment of a disease in which an increased blood concentration and/or a prolonged blood half-life of the endogenous ligand are/is effective for the prophylaxis and/or treatment thereof,

[0014] [15] the ageut of [14] above, wherein the disease is selected from the group consisting of metabolic disease, bone and joint disease, cardiovascular disease, cranial nerve disease, infectious disease, cancer, blood disorder, urologic disease, infertility/erectile dysfunction, deficient growth and immunodeficiency,

[0015] [16] a method for the prophylaxis and/or treatment of a disease in a mammal, whereiu an increased blood concentration aud/or a prolonged blood half-life of an endogenous ligand are/is effective for the prophylaxis and/or treatment of the disease, which method comprises administering to the mammal an effective amount of an antibody that has an affinity to the endogenous ligand but does not ueutralize the same substantially, without administering a compound the same as or substantially the saue as the endogenous ligand, so as to increase the blood stability of the endogenous ligand, thereby enhancing a receptor activity-regulatory action of the ligand, and

[0016] [17] a use of an antibody that has an affinity for an endogenous ligand but does not neutralize the same substantially for the manufacture of an agent for the prophylaxis and/or treatment of a disease in which an increased blood conceutration and/or a prolonged blood half-life of the endogenous ligaud are/is effective for the prophylaxis and/or treatment thereof, and the like.

[0017] When administered to an animal, the antibody of the present invention is capable of bindiug to an endogenous ligand and stabilizing the same to raise the blood ligand concentration and, as a result, to enhance the receptor activity-regulatory action of the ligand because of its characteristic of having affinity for an endogeuous ligands but not completely neutralizing the same. Also, because the antibody of the present inventiou exhibits its effect in an amount sufficient to capture a ligand that is present essentially only in trace amounts in blood, it permits a remarkable reduction in clinical doses compared with existing antibody medicines and enables the provision of a safer and less expensive preparation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 shows the binding activities of various anti-PACAP monoclonal antibodies [(a) PA-1Na; (b)

PA-3Na; (c) PA-5Na; (d) PA-6Na; (e) PA-2Ca; (f) PA-1Ca] against PACAP38, partial peptides thereof, and VIP. The ordinate indicates [measured amount of label (B)]/[amount of label without addition of competitive peptide (B_0)]× 100(%); the abscissa indicates the concentration (M) of each competitive peptide. —○—: PACAP38NH_2; —●—: PACAP27NH_2; —▲—: PACAP(4-27)OH; —■—: PACAP(1-13)OH; —x—: VIP; —Δ—: PACAP(14-38)NH_2; —□—: PACAP(31-38)NH_2

[0019] FIG. 2 is a schematic diagram showing the antigen recognition sites of various anti-PACAP monoclonal antibodies. The upper box indicates PACAP38NH_2, and the numerical figures thereon show amino acid numbers. The arrowhead indicates the processing site of PACAP27. The white portions of the box indicate the amino acid sequences common to VIP, and the hatched portions indicates the amino acid sequences differing from VIP.

[0020] FIG. 3 shows the neutralizing activities of various anti-PACAP monoclonal antibodies on PACAP38NH_2. The ordinate indicates cAMP concentration (pmol/10^5 cells); the abscissa indicates antibody concentration (nM). —○—: PA-1Na; —Δ—: PA-3Na; —□—: PA-5Na; —■—: PA-6Na; —▲—: PA-2Ca; —●—: PA-1Ca

[0021] FIG. 4 shows the suppressive effect of the anti-PACAP non-neutralizing monoclonal antibody PA-6Na on PACAP38 degradation by DPP-IV. The ordinate indicates the degree of binding of ^{125}I-PACAP27 to PACAP receptor (the residual radioactivity difference between experiment 1 and experiment 2 is shown as %, with the residual radioactivity measured with the receptor binding site saturated with excess PACAP38 as 0%, and the activity measured in the absence of PACAP38 as 100%); the abscissa indicates the dilution factor of DPP-IV expressing cell membrane fraction. —○—: in the presence of PA-6Na; —●—: in the absence of PA-6Na

[0022] FIG. 5 shows the suppressive effect of the anti-PACAP non-neutralizing monoclonal antibody (PA-6Na) on PACAP38 degradation by recombinant DPP-IV. The ordinate indicates the degree of binding of ^{125}I-PACAP27 to PACAP receptor (ratio of residual radioactivity, with the radioactivity of input ^{125}I-PACAP27 as 100%); the abscissa indicates the dilution factor of DPP-IV. Symbols show respective conditions: —□—: in the presence of PA-6Na and in the absence of DPP-IV inhibitor; —■—: in the co-presence of PA-6Na and DPP-IV inhibitor; —○—: in the absence of antibody and DPP-IV inhibitor; —●—: in the absence of antibody and in the presence of DPP-IV inhibitor.

[0023] FIG. 6 shows the results of a reporter gene assay of anti-GLP-1 antibodies against GLP-1(7-36) amide [FIG. 6A: GLIT2-329(1)24 (non-neutralizing antibody) and FIG. 6B: GLIT1-492(1)2 (neutralizing antibody)]. The ordinate indicates the residual GLP-1 activity when 2 nM GLP-1(7-36)amide was reacted with each concentration of anti-GLP-1 antibody, which activity was standardized with GLP-1 activity, when reacted with the same concentration of anti-crythropoietin monoclonal antibody, as 100%. Each numerical figure on the abscissa shows the molar ratio of anti-GLP-1 antibody concentration to GLP-1(7-36) amide concentration.

[0024] FIG. 7 shows the results of a GLP-1/GLP-1 receptor binding inhibition assay of anti-GLP-1 antibodies against GLP-1(7-36) amide [GLIT2-329(1)24 and GLIT1-492(1)2]. The ordinate indicates the ratio (residual binding activity) of ^{125}I-labeled GLP-1 bound to GLP-1 receptor membrane fraction when 200 pM ^{125}I-labeled GLP-1(7-36)amide was reacted with anti-GLP-1 antibody, which ratio was standardized with the amount of ^{125}I-labeled GLP-1 bound to GLP-1 receptor membrane fraction, when reacted with the anti-PACAP38NH_2 non-neutralizing antibody obtained in Example 3 (PA-6Na), as 100%; each numerical figure on the abscissa shows the molar ratio of anti-GLP-1 antibody concentration to ^{125}I-labeled GLP-1(7-36) amide concentration.

[0025] FIG. 8 shows the suppression effect of an anti-GLP-1 non-neutralizing monoclonal antibody (GLIT2-329(1)24) on the degradation of GLP-1(7-36) amide by DPP-IV. The ordinate indicates the percentage of the amount of residual GLP-1(7-36) amide after DPP-IV digestion, measured by ELISA, which was standardized with the amount of GLP-1(7-36) amide in the absence of the antibody and DPP-IV as 100%. Respective conditions are shown: GLIT2-329(1)24: in the presence of GLIT2-329(1)24; Anti-EPO Ab: in the presence of anti-crythropoietin antibody; None: in the absence of antibody; DPP-IV(+): in the presence of DPP-IV; DPP-IV(−): in the absence of DPP-IV.

[0026] FIG. 9 shows plasma GLP-1(7-36) amide concentrations obtained one day after intraperitoneal administration of each of saline, mouse IgG (20 mg/kg) and the anti-GLP-1 non-neutralizing monoclonal antibody GLIT2-329(1)24 (20 mg/kg) to satiated Wister Fatty rats (3 animals per group).

BEST MODE FOR EMBODYING THE INVENTION

[0027] The antibody used in the agent for improving the blood stability of the present invention is not subject to limitation, as long as it has affinity to an endogenous ligand of a mammal (for example, human, monkey, bovine, horse, swine, sheep, goat, dog, cat, rabbit, rat, mouse, hamster, guinea pig and the like) and substantially does not neutralize the same.

[0028] As used herein, "endogenous" means naturally existing (=inherent) in a living organism (in the present invention, in the body of the mammal as the recipient of the agent for improving the blood stability of the present invention). Therefore, one administered to the animal from outside the body thereof is not included even when it is the same substance as a substance naturally occurring in a living organism.

[0029] "A ligand" means a molecule that is capable of specifically binding to a molecule that is present in cells and has the function of recognizing and transmitting external stimuli (i.e., receptor) to activate (=agonistic) or not to activate (antagonistic) the function of the receptor; examples thereof include, but are not limited to, hormones, cytokines, chemokines, growth factors, hematopoietic factors, neurotransmitters and the like.

[0030] "Substantially does not neutralize" an endogenous ligand means that the biological activity (i.e., receptor activity-regulatory action) of an endogenous ligand molecule is not inhibited at all, or an endogenous ligand molecule is inhibited only to the extent that the endogenous

ligand as a whole can exhibit the biological activity necessary for the living organism. That is, there are some cases in which the desired ligand activity can be exhibited as a whole even if the activity of each endogenous ligand molecule is partially inhibited, because the antibody stabilizes the endogenous ligand to increase the blood ligand concentration, so that the antibody apparently does not neutralize the endogenous ligand. Herein, an antibody that substantially does not neutralize an endogenous ligand is hereinafter sometimes referred to as "a non-neutralizing antibody"

[0031] Preferably, the non-neutralizing antibody used in the present invention has a neutralizing activity of about 80% or less, more preferably about 50% or less, particularly preferably about 20% or less. As used herein, "neutralizing activity" means the percent inhibition of the activity of the endogenous ligand molecule. Ligand activity means receptor activity-regulatory action, and it can be measured with the activity of the receptor (effector activity-regulatory action) as the index for an agonistic ligand, or with the ability to bind to the receptor or the like as the index for an antagonistic ligand. Neutralizing activity can be calculated using the equation below.

$$\text{neutralizing activity } (\%)=([A_{free}]-[A_{bound}])/[A_{free}]\times 100$$

A_{free}: ligand activity in the absence of antibody

A_{bound}: ligand activity in the presence of antibody

[0032] The endogenous ligand targeted by the agent for improving the blood stability of the present invention is not subject to particular limitation, as long as the ligand's biological activity enhanced as a result of its stabilization by an antibody has an effect desirable for the living organism, and it may be any ligand molecule that has a shorter blood half-life than that of the antibody.

[0033] Preferably, the endogenous ligand that can be stabilized by the present invention is exemplified by a peptidic compound. As used herein, "a peptidic compound" means an optionally chosen compound having 1 or 2 or more peptide bonds in the molecule thereof; preferably, "a peptide" or "a protein" resulting from the polymerization of a plurality of amino acids via peptide bonds can be mentioned.

[0034] In the present description, a peptide or protein shown by an amino acid sequence is denoted with the N-terminal (amino terminal) described as the left end and the C-terminal (carboxyl terminal) as the right end, in accordance with the common way of describing peptides. The peptide or protein that is an endogenous ligand in the present invention may have any of a carboxyl group, a carboxylate, an amide or an ester at the C-terminal thereof. When the peptide or protein has a carboxyl group (or carboxylate) at a position other than the C-terminal, the carboxyl group may be amidated or esterified. Furthermore, the peptide and protein may also include a peptide or protein wherein the amino group of the N-terminal amino acid residue is substituted by formyl group, acetyl group and the like, a peptide or protein wherein the N-terminal glutamine residue has been converted to pyroglutamic acid, or a peptide or protein wherein a substitutent (for example, —OH, —SH, amino group, imidazole group, indole group, guanidino group and the like) on a side chain of an amino acid in the molecule is substituted by other substitutent (for example, formyl group, acetyl group and the like).

[0035] Alternatively, complex proteins (peptides) wherein a sugar chain, a fatty acid, a lipid, a nucleic acid and the like are bound to a peptide chain are also encompassed in the scope of peptides or proteins in the present invention.

[0036] As specific examples of preferable peptidic compounds as endogenous ligands, peptides or proteins that function as biologically active substances such as hormones, cytokines, chemokines, growth factors, hematopoietic factors, and neurotransmitters can be mentioned.

[0037] As examples of hormone, glucagon-like peptide-1 (GLP-1), calcitomin, pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), leuteinizing hormone-releasing hormone (LH-RH), melanocyte-stimulating hormone (MSH), ghrelin, erythropoietin (EPO), thrombopoietin (TPO), insulin, growth hormone, leptin, adiponectin, adrenomedullin, tissue plasminogen activator (tPA), single-chain urokinase-type plasminogen activator (scu-PA), two-chain urokinase-type plasminogen activator (tcu-PA), angiotensin I, angiotensin III, angiotensin II inhibitor, bradykinin, corticotropin, dynorphin, kyotorphin, endorphin, enkephalin, secretin, growth hormone-releasing factor (GRF), neurotensin, parathyroid hormone (PTH), oxytocin, vasopressin, vasotocin, somatostatin, thyrotropin-releasing hormone (TRH), thyroid-stimulating hormone, prolactin and the like can be mentioned.

[0038] As examples of hormone cytokine, interleukins (e.g., IL-1 to IL-20), interferons (e.g., IFNα, IFNβ, IFNγ), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), tumor necrosis factor (TNF), lymphotoxin, T-cell replacing factor (TRF), antigen-specific suppressor factor (TsF), soluble immune response suppressor factor (SIRF), suppressor-inducing factor (SIF), macrophage activating factor (MAF), macrophage migration inhibitory factor (MIF), leukocyte migration inhibitory factor (LIF) and the like can be mentioned.

[0039] As examples of chemokine, CXC chemokines such as ENA-8, GCP-2, GRO-α, GRO-β, GRO-γ, BCA-1, IP-10, MIG, PF-4 etc., CC chemokines such as MIP-3α, MIP-3β, eotaxin; eotaxin-2, MCP-1 to 4, MIP-1α, MIP-1β, RANTES etc., C chemokines such as lymphotactin etc., CX, C chemokines such as fractalkin, neurotactin etc. and the like can be mentioned.

[0040] As examples of growth factor, EGF family such as epidermal growth factor (EGF), betacellulin, betacellulin-δ4, transforming growth factor-α (TGF-α), heparin-binding EGF-like growth factor (HB-EGF) etc., PDGF family such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) etc., FGF family such as fibroblast growth factor (e.g., FGF-1 to -9) etc., IGF family such as insulin-like growth factor (e.g., IGF-1, IGF-II) etc., HGF family such as hepatocyte growth factor (HGF) etc., TGF-β family such as transforming growth factor-β (e.g., TGF-β1 to -β5) etc., NGF family such as neurotroplins (e.g., NT-1 to -5) etc., and the like can be mentioned.

[0041] As examples of hematopoietic factor, the above-mentioned EPO, TPO, GM-CSF, G-CSF, M-CSF and the like can be mentioned.

[0042] As examples of neurotransmitter, the above-mentioned dynorphin, kyotorphin, endorphin, enkephalin and the like can be mentioned.

[0043] In a preferred mode of embodiment, the agent for improving the blood stability of the present invention stabilizes a peptidic compound that is an endogenous ligand for a G protein-coupled receptor (GPCR). Examples of the GPCR include, but are not limited to, class A GPCRs such as neuropeptide receptors and chemokine receptors, class B GPCRs such as glucagon receptor, calcitonin receptor, and PTH receptor, and the like. As examples of endogenous ligands for class B GPCRs, peptides or proteins belonging to the secretin/glucagon super family, such as GLP-1, calcitonin, PACAP, and VIP, can be mentioned. As examples of endogenous ligands for GPCRs other than those of class B, LH-RH, metastin, GPR7-specific ligand [also referred to as neuropeptide B (NPB)] and ligand for both GPR7 and GPR8 [also referred to as neuropeptide W (NPW)] (these are herein generically referred to as GPR7/GPR8 ligands), MSH, ghrelin, APJ receptor-specific ligand (referred to as apelin) and the like can be mentioned.

[0044] In another preferred mode of embodiment, the agent for improving the blood stability of the present invention stabilizes a peptide or protein that is an endogenous ligand for receptors except GPCR. As examples of such peptides, ANP, BNP, CNP, beta cellulin, beta cellulin 64, adrenomedullin and the like can be mentioned; as examples of such proteins, EPO, TPO, insulin, interferons, growth hormones, GM-CSF, leptin, adiponectin and the like can be mentioned.

[0045] The above-described peptide or protein may have an amino acid sequence differing from a known amino acid sequence with respect to 1 or 2 or more amino acids, as long as it is endogenous to the recipient mammal and retains the action as a ligand. As used herein, "retains the action as a ligand" means to retain the capability of binding to the receptor, and the degree of agonism/antagonism may differ. Such examples include, but are not limited to, genetic polymorphisms, splicing variants, fragments resulting from post-translational processing, and the like. These are herein generically referred to as "analogs".

[0046] The present invention can be applied to any endogenous ligand other than peptidic compounds, as long as it can produce a non-neutralizing antibody. As such endogenous ligand, for example, ligands to GPCR or nuclear receptor such as non-peptidic hormones including steroid (e.g., glucocorticoid, estradiol, estriol, testosterone, etc.) etc., biological amine (e.g., adrenaline, dopamine, histamine, acetylcholine, noradrenaline, etc.), lipid (e.g., anandamide, cannabinoide, leukotriene, lysophosphatidic acid, platelet-activating factor, etc.), fatty acids (e.g., GPR40 ligand, etc.), eicosanoid (e.g., prostaglandin, thromboxane, etc.), bile acid (e.g., TGR5 ligand, etc.), amino acid or derivative thereof (e.g., metabolic glutamate, GABA, etc.), purine or nucleic acid (e.g., adenosine, cAMP, ATP, UTP, ADP, UDP, etc.) aud the like can be mentioned.

[0047] The agent for improving the blood stability of the present invention is characterized by extending the blood half-life of an endogenous ligand by forming an immune complex with an antibody. For example, the blood half-life of a peptidic endogenous ligand like a biologically active peptide is very short because it is likely to undergo degradation by a peptidase in a living organism, because its renal clearance is fast, and because of other reasons. On the other hand, the blood half-life of an antibody is very long; for example, in the case of a whole antibody molecule, the blood half-life thereof is reportedly normally about 3 weeks. As a biologically active peptide has formed an immune complex with an antibody against the same, factors that destabilize the biologically active peptide, such as peptidases, are prevented by the antibody molecule from approaching to the target cleavage site of the peptide molecule, and the molecular size as the peptide-antibody complex increases so that the peptide is more unlikely to undergo renal clearance; for these and other reasons, the blood stability of the peptide improves [for example, peptides such as GLP-1 and PACAP are cleaved at the N-terminus thereof by dipeptidyl-peptidase-IV (herein sometimes abbreviated as DPP-IV); because the resulting fragments have antagonist activity for the receptors, the agent for improving the blood stability of the present invention, which comprises a non-neutralizing antibody against these peptides, is particularly useful as a suppressor of the degradation of the peptides by DPP-IV].

[0048] Therefore, the agent for improving the blood stability of the present invention can be generally used to improve the blood stability of an endogenous ligand whose blood half-life in the free form is shorter than that of an antibody (to be specific, an endogenous ligand whose blood half-life in the free form is about 1 week or less); preferably, the agent for improving the blood stability of the present invention can be applied to an endogenous ligand whose blood half-life in the free form is about 1 day or less, more preferably about 12 hours or less, still more preferably about 6 hours or less, particularly preferably 2 hours or less, most preferably 1 hours or less and the like.

[0049] Although the antibody used in the present invention may be a monoclonal antibody or a polyclonal antibody, as long as it substantially does not neutralize the endogenous ligand that is an antigen, it is preferable to use a monoclonal antibody for obtaining a non-neutralizing antibody relatively easily because the region where antibody can bind without neutralizing the antigen is normally limited. Although the isotype of the antibody is not subject to limitation, it is preferably IgG, IgM or IgA, particularly preferably IgG.

[0050] The antibody used in the present invention is not subject to limitation, as long as it has at least a complementality determining region (CDR) for specifically recognizing and binding to the target antigen; in addition to the whole antibody molecule, the antibody may, for example, be a fragment such as Fab, Fab', or $F(ab')_2$, a genetically engineered conjugate molecule such as scFv, scFv-Fc, minibody, or diabody, or a derivative thereof modified with a molecule having protein stabilizing action, such as polyethylene glycol (PEG), or the like, and the like.

[0051] When the antibody used in the present invention is a monoclonal antibody, it can be prepared by, for example, the following method.

(1) Preparation of Immunogen

[0052] As the immunogen, a compound having one or 2 or more kinds of the same epitope as the target antigen or derivative thereof can be used. For example, when the target antigen is a peptidic compound such as a peptide or protein, the antigen or derivative thereof can be obtained by (a)

isolating and purifying from an antigen-producing tissue or cells of a mammal, for example, a human, monkey, rat, mouse and the like, by using a method known per se or a method based thereon, (b) chemically synthesizing by a method of peptide synthesis known per se, using a peptide synthesizer and the like (c) culturing a transformant comprising a DNA that encodes the antigen or derivative thereof, or by (d) biochemically synthesizing by using a cell-free transcription/translation system with a nucleic acid that encodes the antigen or derivative thereof as a template.

[0053] (a) When the antigen is prepared from a mammalian tissue or cells, it is possible to isolate and purify the antigen by homogenizing the tissue or cells, thereafter performing extraction with an acid or alcohol and the like, and subjecting the extract to a protein separation technique known per se (e.g., salting out, dialysis, chromatographies such as gel filtration chromatography, reversed phase chromatography, ion-exchange chromatography, and affinity chromatography, and the like). The antigen peptide (protein) obtained can be used as the immunogen as is, and can also be used as the immunogen in the form of a partial peptide prepared by limited degradation using a peptidase and the like.

[0054] (b) When the antigen or a fragment or derivative thereof is chemically synthesized, the synthetic peptide is exemplified by ones having the same structure as the above-described antigen peptide (protein) purified from naturally occurring substances; to be specific, a peptide comprising 1 or 2 or more kinds of the same amino acid sequence as the amino acid sequence at an optionally chosen portion comprising 3 or more, preferably 6 or more, amino acids in the amino acid sequence of the antigen peptide (protein) and the like are used.

[0055] (c) When the antigen or a fragment or derivative thereof is produced using a transformant comprising a DNA, the DNA can be prepared according to a known cloning method [for example, the method described in Molecular Cloning (2nd ed.; J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989) and the like]. As the cloning method, for example, a method for (i) isolating a DNA that encodes the antigen from a cDNA library by the hybridization method using DNA probes designed on the basis of the gene sequence that encodes the antigen peptide (protein), (ii) preparing a DNA that encodes the antigen or a fragment thereof by a PCR method using DNA primers designed on the basis of the gene sequence that encodes the antigen peptide (protein) with a cDNA as the template, and inserting the DNA into an expression vector matching a host, thereafter transforming the host with the expression vector, and culturing the thus-obtained transformant in a suitable medium, and the like can be mentioned.

[0056] (d) When a cell-free transcription/translation system is utilized, a method for synthesizing an mRNA by using an expression vector incorporating a DNA that encodes the antigen or a fragment thereof (for example, an expression vector wherein the DNA is placed under the control of the T7 or SP6 promoter and the like, and the like) as the template, that was prepared by the same method as (c) above, a transcription reaction mixture comprising an RNA polymerase matching the promoter, and its substrates (NTPs); and thereafter performing a translation reaction with the mRNA as the template using a known cell-free translation system (e.g., *E. coli*, rabbit reticulocytes, extract from wheat germ etc.), and the like can be mentioned. By adjusting the salt concentration and the like appropriately, the transcription reaction and the translation reaction can also be carried out in the same reaction mixture at one time.

[0057] As the immunogen, a whole antigen peptide (protein) molecule or a peptide having a partial amino acid sequence can be used. When a whole antigen peptide is used as the immunogen, selection is performed with neutralization activity and antigenic determinant mapping as the indexes. On the other hand, when antibodies recognizing a particular epitope are systemically generated and selected with a neutralizing activity as the index, a peptide having a partial amino acid sequence of an antigen peptide (protein) is used as the immunogen.

[0058] As examples of the partial amino acid sequence, those comprising 3 or more continuous amino acid residues, preferably those comprising 4 or more, more preferably 5 or more, still more preferably 6 or more continuous amino acid residues, can be mentioned. Alternatively, as examples of the amino acid sequence, those comprising 20 or less continuous amino acid residues, preferably those comprising 18 or less, more preferably 15 or less, still more preferably 12 or less continuous amino acid residues, can be mentioned. A portion of these amino acid residues (e.g., 1 to several residues) may be substituted with a substitutent group (e.g., Cys, hydroxyl group, etc.). The peptide used as the immunogen has an amino acid sequence comprising one to several such partial amino acid sequences.

[0059] Such a peptide can be produced in accordance with the methods (b) to (d) above, or by cleaving an antigen peptide (protein) or a derivative thereof, prepared by the methods (a) to (d) above, with a suitable peptidase and the like; however, to prepare peptides having various partial amino acid sequences systematically, it is preferable to use a known method of peptide synthesis.

[0060] The method of peptide synthesis may, for example, be any of solid phase synthesis and liquid phase synthesis. The desired peptide can be produced by condensing a partial peptide or amino acids that can constitute the peptide and the remaining portion, and removing the protecting group when the product has a protecting group. Condensation and removal of protecting group can be achieved by known methods, for example, the methods described in (1) or (2) below.

(1) M. Bodanszky and M. A. Ondetti: Peptide Synthesis, Interscience Publishers, New York (1966)

(2) Schroeder and Luebke: The Peptide, Academic Press, New York (1965)

[0061] And after the reaction, the peptide can be purified and isolated using conventional methods of purification, such as solvent extraction, distillation, column chromatography, liquid chromatography, recrystallization, etc., in combination thereof. When the peptide obtained by the above-mentioned method is in a free form, it can be converted to a suitable salt by a known method; conversely, when the peptide is obtained in the form of a salt, the salt can be converted to a free form or other salt by a known method.

[0062] For an amide form of peptide, commercially available resins for protein synthesis, which are suitable for

amide formation, can be used. As examples of such resins, chloromethyl resiu, hydroxymethyl resiu, benzhydrylamine resin, aminomethyl resin, 4-benzyloxybenzyl alcohol resin, 4-methylbenzhydrylamine resiu, PAM resin, 4-hydroxymethylmethylphenyl acetamidomethyl resin, polyacrylamide resin, 4-(2',4'-diuethoxyphenyl-hydroxymethyl)phenoxy resin, 4-(2',4'-dimethoxyphenyl-Fmoc-aminoethyl)phenoxy resin and the like can be mentioned. Using such resins, amino acids having α-amino groups and side-chain functional groups appropriately protected are condensed on the resin in accordance with the sequence of the desired peptide according to various condensation methods known per se. At the end of the reaction, the peptide is excised from the resin and the various protecting groups are removed simultaneonsly, thus affording the desired peptide. Alternatively, the desired peptide can also be ohtained by nsing chlorotrityl resin, oxime resin, 4-hydroxybenzoic acid resin and the like, excising a partially protected peptide, and further removing protecting groups by a conventional method.

[0063] For the above-described condensation of protected amino acids, various activation reagents for peptide synthesis may be used, with preference given to carbodiimides. As the carbodiimides, DCC, N,N'-diisopropylcarbodiimide, N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide and the like can be mentioned. For activation with these reagents, protected amino acids may be added directly to the resin along with a racemization inhibitor (for example, HOBt, HOOBt etc.), or may be added to the resin after being previously activated to the form of a symmetric acid anhydride, HOBt ester, or HOOBt ester. Solvents to be used in the activation of protected amino acids or condensation with the resin can be selected as appropriate from among the solvents known to be usable for peptide condensation reactions. For example, acid amides such as N,N-dimethylformamide, N,N-dimethylacetamide, and N-methylpyrrolidone; halogenated hydrocarbons such as methylene chloride and chloroform; alcohols snch as trifluoroethanol; snlfoxides such as dimethylsulfoxide; tertiary amines snch as pyridine; ethers such as dioxane and tetrahydrofuran; nitriles such as acetonitrile and propionitrile; esters such as methyl acetate and ethyl acetate; appropriate mixtures of these solvents, and the like can be used. Reaction temperature is selected as appropriate from the range known to be useful for peptide bond formation reactions, and is normally selected as appropriate from the range of abont −20° C. to about 50° C. The activated amino acid derivative is normally used in an excess of about 1.5 to abont 4 times. If a test nsing the ninhydrin reaction reveals insufficient condensation, the condensation can be completed by repeating the condensation reaction without splitting off the protecting groups. If the condensation is yet insufficient even after repeating the reaction, unreacted amino acids can be acetylated with acetic anhydride or acetylimidazole so that an influence on the snbsequent reactions can be avoided.

[0064] Protection and protecting groups for the functional groups that should not involve the reaction of the starting amino acid, splitting off the protecting groups, activation of the functional groups involved in the reaction, and the like can be selected as appropriate from among known gronps or known means.

[0065] Examples of the protecting groups for the amino groups of the starting amino acid include Z, Boc, tertiary pentyloxycarbonyl, isobornyloxycarbonyl, 4-methoxybenzyloxycarbonyl, Cl-Z, Br-Z, adamantyloxycarbonyl, trifluoroacetyl, phthaloyl, formyl, 2-nitrophenylsulphenyl, diphenylphosphinothioyl, Fmoc and the like. As carboxyl-protectmg group, for example, C_{1-6} alkyl group, C_{3-8} cycloalkyl group, C_{7-14} aralkyl gronp, 2-adamantyl, 4-nitrobenzyl, 4-methoxybenzyl, 4-chlorobenzyl, phenacyl and benzyloxycarbonyl hydrazide, tertiary bntoxycarbonyl hydrazide, trityl hydrazide and the like can be mentioned.

[0066] The hydroxyl group of serine or threonine can be protected by, for example, esterification or etherification. Examples of gronps suitable for this esterification include lower (C_{1-6}) alkanoyl groups such as acetyl group, aroyl groups snch as benzoyl gronp, groups derived from carbonic acid, such as benzyloxycarbonyl gronp and ethoxycarbonyl group, and the like. As examples of groups suitahle for the etherification, benzyl gronp, tetrahydropyranyl gronp, t-butyl group and the like can be mentioned.

[0067] Examplcs of the protecting gronp for the phenolic hydroxyl group of tyrosine include Bzl, Cl-Bzl, 2-nitrobenzyl, Br-Z, tertiary bntyl and the like.

[0068] Examples of the protecting group for the imidazole moiety of histidine include Tos, 4-methoxy-2,3,6-trimethylbenzenesulfonyl, DNP, Bom, Bum, Boc, Trt, Fmoc and the like.

[0069] Examples of activated carboxyl groups in the starting material inclnde corresponding acid anhydrides, azides, activated esters (esters with alcohols (for example, pentachlorophenol, 2,4,5-trichlorophenol, 2,4-dinitrophenol, cyanomethyl alcohol, para-nitrophenol, HONB, N-hydroxysnccimide, N-hydroxyphthalimide, HOBt)]. Examples of activated amino groups in the starting material include corresponding phosphoric amides.

[0070] As examples of the method used to remove (split off) the protecting group, catalytic reduction in a hydrogen gas stream in the presence of a catalyst such as Pd-black or Pd-carbon; acid treatment with anhydrous hydrogen fluoride, methanesulfonic acid, trifluoromethanesulfonic acid or triflnoroacetic acid, or a mixed solution thereof; treatment with a base such as diisopropylethylamiue, triethylamine, piperidine or piperazine; reduction with sodium in liqnid ammonia, and the like can be mentioned. The reaction of splitting of the protecting gronp by the above-described acid treatment is normally performed at a temperature of about −20° C. to 40° C.; in the acid treatment, addition of a cation scavenger such as anisole, phenol, thioanisole, meta-cresol, para-cresol, dimethylsulfide, 1,4-butanedithiol or 1,2-ethanedithiol is effective. The 2,4-dinitrophenyl group used as the protecting group for the imidazole moiety of histidine is removed by thiophenol treatment; the formyl gronp nsed as the protecting group for the indole moiety of tryptophan is removed by alkali treatment with dilnte sodinm hydroxide solution, dilnte ammonia or the like, as well as by the above-described acid treatment in the presence of 1,2-ethanedithiol, 1,4-bntanedithiol or the like.

[0071] In another method of obtaining an amide of the peptide, for example, the α-carboxyl group of the carboxy-terminal amino acid is first protected by amidation, and the peptide chain on the amino group side is then extended to a desired length; thereafter, a peptide having only the protecting gronp for the N-terminal α-amino group in the peptide chain removed and a peptide having only the protecting

group for the C-terminal carboxyl group removed are prepared, and the two peptides are condensed in a mixed solveut as described above. Details of the condensation reaction are the same as those described above. After the protected peptide obtained by the condensation is purified, all the protecting groups are removed by the above-described method to give the desired crude peptide. This crude peptide may be purified by various known means of purification, and the major fraction uay be lyophilized to give au amide of the desired peptide.

[0072] An ester of the peptide can be obtained by, for example, condensing the α-carboxyl gronp of the carboxy-termiual amino acid with a desired alcohol to prepare an amino acid ester, and then following the same procedures as those for the amide of the peptide.

[0073] For example, as meutioned in the Examples to be mentioned later, when PACAP38NH_2 or a partial peptide of PACAP38NH_2 is synthesized by the solid phase methods, using any of the insoluble resins known in the art such as chloromethyl resins, 4-methylbenzhydrylamine resins and 4-oxymethylphenylacetamidomethyl resins, protected amino acids are successively condensed to the C-terminal side of PACAP38NH_2 or of the partial peptide of PACAP38NH_2 according to method known in the art. Then, all protecting groups are removed by hydrogen fluoride treatment, followed by purification by methods known in the art such as high performance liquid chromatography, whereby the desired PACAP38NH_2 or partial peptide of PACAP38NH_2 can be obtained.

[0074] For example, the N-protected amino acids can be produced by protecting α-amino groups with Boc groups (to be expressed as Boc-Xaa), the hydroxyl groups of serine and threonine with Bzl groups (to be expressed as Ser(Bzl)), the ω-carboxylic acid groups of glutamic acid and aspartic acid with OBzl groups (to be expressed as Glu(OBzl)), the ε-amino group of lysine with a Cl-Z group (to be expressed as Lys(Cl-Z)), the hydroxy gronp of tyrosine with a Br-Z group (to be expressed as Tyr(Br-Z)), the guanid group of arginine with a Tos group (to be expressed as Arg(Tos)), and the imidazole group of histidine with a Tos group (to be expressed as His(Tos)).

[0075] The antigen permit direct use for immunization in an insolubilized form, as loug as it has immnnogenicity; when an antigen of low molecular weight (for example, molecular weight about 3,000 or less) having only one to several antigenic determinants in the molecnle thereof (for example, the above-described peptide and the like) is nsed, it can be used for immunization in the form of a complex bound or adsorbed to a suitable carrier becanse these antigens are normally hapten molecules of low immunogenicity. As the carrier, a naturally occurring or synthetic polymer can be used. As examples of the naturally occurring polymer, serum albumin of a mammal snch as bovine, rabbit, or human, thyroglobulin of a mammal such as bovine or rabbit, ovalbumin of chicken, hemoglobin of a mammal such as boviue, rabbit, human, or sheep, keyhole limpet hemocyanin (KLH) and the like can be nsed. As examples of the synthetic polymer, various latexes of polymers or copolymers of polyanino acids, polystyrenes, polyacryls, polyvinyls, polypropylenes and the like, and the like can be mentioned. Regarding the mixing ratio of the carrier and hapten, any combiuation in any ratio can be bound or adsorbed, as long as an antibody against the autigeu bound or adsorbed to the carrier is prodnced efficiently; usually, one wherein the above-described naturally occurring or synthetic polymer carrier in common use in preparing au antibody against hapten is bound or adsorbed in a ratio by weight of 0.1 to 100 to 1 of hapten can be used.

[0076] Various condensing agents can be used for coupling the hapten and carrier. For example, diazonium compounds such as bisdiazotized benzidine, which crosslink tyrosine, histidine, and tryptophan; dialdehyde componnds such as glutaraldehyde, which crosslink amiuo groups together; diisocyanate componnds such as tolnene-2,4-diisocyauate; dimaleimide compounds such as N,N'-o-phenylcnedimalcimide, which crosslink thiol groups together; maleimide activated ester componnds, which crosslink amino groups and thiol groups; carbodiimide componnds, which crosslink amino groups and carboxyl groups; and the like can be used advantageously. When amino groups are crosslinked together, it is also possible to react one amino gronp with an activated ester reageut having a dithiopyridyl gronp (for example, SPDP and the like), followed by reduction, to introduce the thiol gronp, and to introduce a maleimide group into the other amino group using a maleimide activated ester reagent, followed by a reaction of both.

(2) Preparation of Monoclonal Antibody

[0077] An antigen is administered as is, or along with a carrier or a diluent, to a warm-blooded animal at a site enabling antibody production by the methods such as intraperitoneal injection, intravenous injection, subcutaneous injection, intradermal injection and the like. In order to increase antibody prodnctivity upon the administration, Freund's complete adjuvant or Freund's incomplete adjuvant may be administered. Dosing is normally performed about two to 10 times in total every 1 to 6 weeks. As examples of the warm-blooded animal used, monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, donkeys and chickens can be mentioned. Although it is preferable to use a mammal of the same species as the recipient in order to avoid the problem of anti-lg antibody production, mice and rats are generally preferably used for generating a monoclonal antibody.

[0078] Because artificial immunization to humans is ethically difficult, it is preferable, when the agent for improving the blood stability of the preseut invention targets a human, (i) to obtain a human antibody by immnnizing a hnman antibody-prodncing aninal (e.g., monse) produced according to a method described below, (ii) to produce a chimeric antibody, humanized antibody or fully human antibody according to a method described below, or (iii) to obtain a human antibody nsing in combination the in vitro immnnization method and cell immortalization with virus, hnman-human (or -monse) hybridoma prodnction technique, phage display method and the like. Note that the in vitro immunization method can also be used preferably as a method for obtaining an antigen against an antigen that is nnstable and difficult to prepare in large amounts for the pnrpose of preparing a non-human animal-derived antibody, because there is the possibility of obtaining an antibody against an antigen for which antibody production is snppressed by ordinary immunization, because it is possible to obtain an antibody with an amount of antigen on the nanogram to microgram order, because immunization completes in several days, and for other reasons.

[0079] As the animal cells used in the in vitro immunization method, lymphocytes, preferably B-lymphocytes and the like, isolated from peripheral blood, spleen, lymph node and the like of a human and the above-described warm-blooded animals (preferably mouse or rat) can be mentioned. For example, in the case of mouse or rat cells, the spleen is extirpated from an about 4- to 12-week-old animal, and splenocytes are separated and rinsed with a appropriate medium [e.g., Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium, Ham's F12 medium and the like], after which the splenocytes are suspended in an antigen-containing medium supplemented with fetal calf serum (FCS; about 5 to 20%) and cultured using a CO_2 incubator and the like for about 4 to 10 days. Examples of the antigen concentration include, but are not limited to, 0.05 to 5 μg. It is preferable to prepare a culture supernatant of thymocytes of an animal of the same strain (preferably at about 1 to 2 weeks of age) according to a conventional method, and to add the supernatant to the medium.

[0080] Because it is difficult to obtain a thymocyte culture supernatant in in vitro immunization of human cells, it is preferable to perform immunizatiou by adding, to the medium, several kinds of cytokines such as IL-2, IL-4, IL-5, and IL-6 and the like, aud if necessary, an adjuvant substance (e.g., muramyldipeptide and the like) along with the antigen.

[0081] In preparing a monoclonal antibody, it is possible to establish au antibody-producing hybridoma by selecting an individual or cell population showiug an elevated antibody titer from among antigen-immunized warm-blooded animals (e.g., mice, rats) or animal cells (e.g., human, mouse, rat), respectively; collecting spleens or lymph nodes at 2 to 5 days after the final immunization or collecting the cells after 4 to 10 days of cultivation after in vitro immunization to isolate antibody-producing cells; and fusing the isolated cells with myeloma cells. A measurement of serum antibody titer can be performed by, for example, reacting a labeled antigen and an antiserum, and thereafter determining the activity of the label bound to the antibody.

[0082] Althongh the myeloma cells are not snbject to limitation, as long as they are capable of producing a hybridoma that secretes a large amount of antibody, those that do not produce or secrete the antibody per se are preferable, with greater preference given to those of high cell fusion efficiency. To facilitate hybridoma selection, it is preferable to use a cell line that is snsceptible to HAT (hypoxanthine, aminopterin, thymidine). As examples of the mouse myeloma cells, NS-1, P3U1, SP2/0, AP-1 and the like can be mentioned; as examples of the rat myeloma cells, R210.RCY3, Y3-Ag 1.2.3 and the like can be mentioned; as examples of the human myeloma cells, SKO-007, GM 1500-6TG-2, LICR-LON-HMy2, UC729-6 and the like ean be mentioned.

[0083] Fusion operation can be performed according to a known method, for example, the method of Koehler and Milstein [*Nature,* 256, 495 (1975)]. As a fusion promoter, polyethylene glycol (PEG), Sendai virus and the like ean be mentioned, and PEG and the like are preferably nsed. Although the molecular weight of PEG is not subject to limitation, PEG1000 to PEG6000, which are of low toxicity and relatively low viscosity, are preferable. As examples of the PEG concentration, about 10 to 80%, preferably about 30 to 50%, can be mentioned. As the solution for diluting PEG, varions bnffers such as serum-free medium (e.g., RPMI1640), complete medium comprisiug about 5 to 20% serum, phosphate bnffered saline (PBS), and Tris buffer can be used. DMSO (e.g., about 10 to 20%) can also be added as desired. As examples of the pH of the fusion solution, about 4 to 10, preferably about 6 to 8 can be mentioned.

[0084] The ratio by number of antibody-producing cells (splenocytes) and myeloma cells is preferably about 1:1 to 20:1, and the cell fusion can be efficiently performed by incubation normally at 20 to 40° C., preferably at 30 to 37° C., normally for 1 to 10 minutes.

[0085] An antibody-produciug cell liue can also be obtained by infecting antibody-producing cells with a virus capable of transforming lymphocytes to immortalize the cells. As such viruses, for example, Epstein-Barr (EB) virus and the like can be mentioned. Although the majority of persons have immunity because they have ever been iufeeted with this virus in an asymptomatic infection of infectious monouucleosis, virion is also produced when the ordinary EB virus is used; therefore, appropriate purification must be performed. As an EB system free from the possibility of viral contamination, it is also preferable to use a recombinant EB virus that retains the capability of immortalizing B lymphocytes but lacks the capability of replicating virion (for example, deficiency of the switch gene for transition from latent infection state to lytic infection state and the like).

[0086] Because marmoset-derived B95-8 cells secrete EB virus, B lymphocytes can be easily transformed by using a culture supernatant thereof. An antibody-producing B cell line can be obtained by, for example, culturiug these cells using a medium supplemented with serum and penicillin/streptomycin (P/S) (e.g., RPMI1640) or a serum-free medium snpplemented with a cell growth factor, thereafter separating the culture supernatant by filtration or centrifugation and the like, snspending therein antibody-producing B lymphocytes at a suitable concentration (e.g., abont 10^7 cells/mL), and incubating the suspension normally at 20 to 40° C., preferably at 30 to 37° C., normally for abont 0.5 to 2 hours. When human antibody-producing cells are provided as mixed lymphocytes, it is preferable to previously remove T lymphocytes by allowing them to form an E rosette with, for example, sheep erythrocytes and the like, to increase transformation freqnency of EB virus, becanse the majority of persons have T lymphocytes which exhibit cytotoxicity to cells infected with EB virus. It is also possible to select lymphocytes specifie for the target antigen by mixing sheep erythrocytes, previonsly bound with a soluble antigen, with antibody-producing B lymphocytes, and separating the rosette nsing a density gradient of percoll and the like. Furthermore, because antigen-specific B lymphocytes are capped by adding the antigen in large excess so that they no longer present IgG to the surface, mixing with sheep erythroeytes bound with anti-IgG antibody results in the formation of rosette only by antigen-nonspecific B lymphocytes. Therefore, by collecting a layer of eells that don't form rosette from this mixture using a density gradient of percoll and the like, it is possible to select antigen-specific B lymphocytes.

[0087] Human antibody-secreting cells having acquired the capability of proliferating indefinitely by the transfor-

matiou can be back fused with mouse or human myeloma cells in order to stably sustain the antibody-secreting ability. As the myeloma cells, the same as those described above can be used.

[0088] Hybridoma screening and breeding are normally performed using a medium for animal cells (e.g., RPMI1640) containing 5 to 20% FCS or a serum-free medium supplemented with cell growth factors, with the addition of HAT (hypoxanthine, aminopterin, thymidine). As examples of the concentrations of hypoxanthine, aminopterin aud thymidine, about 0.1 mM, about 0.4 μM and about 0.016 mM and the like, respectively, can be mentioned. For selecting a human-mouse hybridoma, ouabain resistance can be used. Because human cell lines are more susceptible to ouabain than mouse cell lines, it is possible to eliminate unfused human cells by adding ouabain at abont 10^{-7} to 10^{-3} M to the medinm.

[0089] In selecting a hybridoma, it is preferable to nse feeder cells or culture snpernatants of certain cells. As the feeder cells, an allogenic cell species having a lifetime limited so that it dies after helping the emergence of hybridoma, cells capable of produciug large amounts of a growth factor useful for the emergence of hybridoma with their proliferation potency reduced by irradiation and the like, and the likc are usod. For cxample, as the mouse fcoder cells, splenocytes, macrophage, blood, thymocytes and the like can be mentioned; as the human feeder cells, peripheral blood mononuclear cells and the like can be mentioned. As examples of the cell culture snpernatant, primary cnlture supernatants of the above-described various cells and culture supernatants of various established cell lines can be mentioned.

[0090] Moreover, a hybridoma can also be selected by reacting a fluorescein-labeled antigen with fusion cells, and thereafter separating the cells that bind to the antigen using a fluorescence-activated cell sorter (FACS). In this case, efforts for cloning can be lessened significantly because a hybridoma that produces an antibody against the target antigen can be directly selected.

[0091] For cloning a hybridoma that prodnces a monoclonal antibody against the target antigen, various methods can be used.

[0092] It is preferable to remove aminopterin as soon as possible because it inhibits many cell functions. In the case of mice and rats, aminopterin can be removed 2 weeks after fusion and beyond because most myeloma cells die within 10 to 14 days. However, a human hybridoma is normally maintained in a medium supplemented with aminopterin for abont 4 to 6 weeks after fusion. It is desirable that hypoxanthine and thymidine be removed more than one week after the removal of aminopterin. That is, in the case of monse cells, for example, a complete medium (e.g., RPMI1640 supplemented with 10% FCS) supplemented with hypoxanthine and thymidine (HT) is added or exchanged 7 to 10 days after fusion. About 8 to 14 days after fusion, visible elones emerge. Provided that the diameter of clone has reached about 1 mm, the amount of antibody in the cnlture supernatant can be measured.

[0093] A measurement of the amount of antibody can be performed by, for example, a method comprising adding the hybridoma culture supernatant to a solid phase (e.g., microplate) to which the target antigeu or a derivative thereof or partial peptide thereof (including the partial amino acid sequence used as the epitope) is adsorbed directly or with a carrier, subsequently adding an anti-immunoglobulin (IgG) antibody (an antibody against IgG derived from an animal of the same species as the animal from which the original antibody-producing cells are derived is used) or protein A, which had been labeled with a radioactive substance (e.g., ^{125}I, ^{131}I, ^{3}H, ^{14}C), enzyme (e.g. β-galactosidase, β-glucosidase, alkaline phosphatase, peroxidase, malate dehydrogenase), fluorescent substance (e.g., fluorescamine, fluorescein isothiocyanate), luminescent substance (e.g., luminol, luminol derivative, luciferin, lucigenin) and the like, and detecting the antibody against the target antigen (epitope) bound to the solid phase, a method comprising adding the hybridoma culture supernatant to a solid phase to which an anti-IgG antibody or protein A is adsorbed, adding the target antigen, a derivative thereof, or a partial peptide thereof labeled with the same labeling reagent as described above, and detecting the antibody against the target antigen (epitope) bound to the solid phase and the like.

[0094] Although limiting dilntion is normally used as the cloning method, cloning using soft agar and cloning using FACS (described above) are also possible. Cloning by limiting dilntion can be performed by, for cxample, thc following procedures, which, however, are not to be construed as limiting.

[0095] The amount of antibody is measured as described above, and positive wells are selected. Selected suitable feeder cells arc previously addcd to a well platc. Cells arc collected from the antibody-positive wells and suspended in complete medium (e.g., RMP11640 snpplemented with 10% FCS and P/S) to obtain a density of 30 cells/mL; 0.1 mL (3 cells/well) of this suspeusion is added to the 96-well plate with feeder cells added thereto; a portion of the remaining cell suspension is diluted to 10 cells/mL and sown to other wells (1 cell/well)in thc same way; the still remaining cell suspension is dilnted to 3 cells/mL and sown to other wells (0.3 cells/well). The cells are cultured for about 2 to 3 weeks until a visible clone appears, when the amount of antibody is measured to select positive wells, and the selected cells are recloned in the same way. In the case of human cells, cloning is relatively difficult, so that a plate in which cells are seeded at 10 cells/well is also prepared. Although a monoclonal antibody-producing hybridoma can be obtained nonnally by two times of subcloning, it is desirable to repeat recloning regularly for several more months to confirm the stability thereof.

[0096] Hybridomas can be cultured in vitro or in vivo.

[0097] As a method of in vitro culture, a method comprising gradually scaling up a monoclonal antibody-producing hybridoma obtained as described above, from a well plate, while keeping the cell density at, for example, abont 10^5 to 10^6 cells/mL, and gradually lowering the FCS concentration, can be mentioned.

[0098] As a method of in vivo cnlture, for example, a method comprising an intraperitoneal injection of a mineral oil to a mouse (a monse that is histocompatible with the parent strain of the hybridoma) to induce plasmacytoma (MOPC) 5 to 10 days later, to which intraperitoneally injecting about 10^6 to 10^7 cells of hybridoma, and collecting ascites fluid under anesthesia 2 to 5 weeks later, can be mentioned.

[0099] Separation and purification of the monoclonal antibody are performed accordiug to a method of immunoglobulin separation and purification [e.g., salting-out, alcohol precipitation, isoelectric point precipitation, electrophoresis, adsorption-desorptiou with an ion exchanger (e.g., DEAE, QEAE), ultracentrifugation, gel filtration, specific purification comprising selectively collecting the antibody by means of an antigen-coupled solid phase or an active adsorbent such as protein A or protein G, and dissociating the linkage to obtain the antibody, and the like] in the same manner as an ordinary separation and purificatiou of the polyclonal autibody.

[0100] As described above, a monoclonal antibody can be produced by culturing a hybridoma in or outside the living body of a warm-blooded animal, and harvesting an antibody froun the body fluid or culture thereof.

[0101] Because the antibody used in the present invention must be one that substantially does not neutralize the endogeenous ligaud as the antigen, it is necessary to examine the degree of neutralization activity of the monoclonal antibody obtaiued. The neutralization activity can be measured by compariug the effector activity-regulatory action of a receptor for the ligand, or ligaud-receptor binding ability, between in the preseuce and abseuce of the antibody. For example, when the endogenous ligand is for a GPCR that couples with G protein, which promotes or suppresses adenylate cyclase (AC) activity, the neutralization activity cau be measured by, in the presence of the ligand alone and in the presence of a ligand-antibody complex using, for example, 1) a method comprising adding ATP to cells or a membrane fraction thereof, comprisiug the GPCR and AC ou the cell membrane thereof, and measuring the amount of resulting cAMP by a competitive immunoassay with cAMP labeled with a radioactivc substance (e.g., ^{125}1), enzyme (e.g., alkaline phosphatase, peroxidase), fluorescent substance (e.g., FITC, rhodamine) and the likc, using an anti-cAMP antibody, 2) a method comprising adding [α-^{32}P]ATP to the above-described cells or a membrane fractiou thereof, separating the resulting [^{32}P]cAMP using an alumiua column and the like, and thereafter measuring the radioactivity thereof, 3) a method comprisiug measuriug the expression level of a reporter gene (e.g., luciferase gene) in transformant cells trausfected with the gene under the control of a cAMP responsive elemeut (CRE), 4) a method comprising adding [^{35}S]GTPγS (a GTP analog not undergoing hydrolysis by the GTPase activity of G protein α-subunit) to a membrane fraction comprising the GPCR, and measuring the radioactivity bouud to the membraue, 5) a method comprising measuring the binding of the GPCR and the ligand by a competitive immunoassay with the ligand labeled with a radioactive substance (e.g., ^{125}1), enzyme (e.g., alkaline phosphatase, peroxidase), fluorescent substance (e.g., FITC, rhodamiue) and the like, and the like. When the receptor for the endogenous ligand is a GPCR that has phospholipase C (PLC) as the effector, there can be used, in place of the above-described methods 1) to 3), 1) a method comprising addiug phosphatidyliuositol-4,5-bisphosphate to cells or a membrane fraction thereof, comprising the GPCR and PLC in the cell membrane thereof, and measuring the amount of the resultiug inositol phosphate, 2) a method comprising measuring the amount of intracellular Ca^{2+} in cells comprisiug the GPCR and PLC in the cell membrane thereof, 3) a method comprising measuring the expression level of a reporter-gene, in transformant cells transfected with the reporter gene under the control of TPA (12-O-tetradecanoylphorbol-13-acetate) respousive element (TRE), whieh is upregulated by Ca^{2+}, and the like. Note that the amount of intracellular Ca^{2+} can be measured spectroscopically using a fluorescent probe (fura-2, indo-1, fluor-3, Caleium-Greeu 1 and the like) or can be measured usiug aequorin which is a calcium-sensitive photoprotein, aud the like. As an apparatus suitable for the spectroscopic measurement using a fluoresceut probe, the FL1PR (Molecular Devices Company) system can be mentioned.

[0102] As a result of performing the above-described assay, for example, an antibody having a neutralization activity of about 80% or less, preferably about 50% or less, more preferably about 20% or less, can be selected as a candidate for the non-neutralizing antibody used in the present invention.

[0103] In a preferred mode of embodiment, because the agent for improving the blood stability of the present inveution is a pharmaceutical product having humans as the subject of administration thereof, the antibody used in the present invention (preferably a mouoclonal antibody) is an antibody whose risk of showiug antigenicity when administered to a human has been reduced; to be specific, the antibody is a fully human autibody, a humanized antibody, a mouse-human chimeric antibody aud the like, particularly preferably a fully human antibody. A humanized antibody and a chimeric antibody can be prepared by genetic engineering technology according to thc method dcscribed below. Although a fully human antibody can also be produced from the above-described human-human (or -mouse) hybridoma, it is desirable to produce it usiug a human antibody-producing animal described below (e.g., mouse) or the phage display method in order to stably supply the autibody in large amounts at low costs.

(1) Preparatiou of Chimeric Antibody

[0104] As used hereiu, "a chimeric antibody" means an antibody whereiu the sequences of the variable regions of the H chain and L chain (V_H and V_L) thereof are derived from a mammalian specics, and wherein the sequences of the constant regions (C_H and C_L) are derived from another mammalian species. The sequences of the variable regions are preferably derived from, for example, au animal species permitting easy preparation of a hybridoma, such as mouse, and the sequences of the constant regions are preferably derived from the recipieut mammalian species.

[0105] As examples of the method of preparing a chimeric antibody, the method described in U.S. Pat. No. 6,331,415 or a partially modified method thereof and the like cau be mentioned. To be specific, first, mRNA or total RNA is prepared from a monoclonal antibody-producing hybridoma (for example, mouse-mouse hybridoma) obtained as described above, according to a conventional method, to synthesize cDNA. DNAs that encode V_H and V_L are amplified and purified by PCR according to a conventional method with thc cDNA as the template, using appropriate primers [for example, oligo DNAs comprising the base sequences that encode the N-terminal sequences of V_H and V_L, respectively, as the seuse primers, and oligo DNAs that hybridize to the base sequences that encode the terminal sequences of C_H and C_L, respectively, as the antisense primer (see, for example, *Bio/Technology*, 9: 88-89, 1991)]. In the same manner, DNAs that eucode C_H aud C_L are

amplified and purified from an RNA prepared from lymphocytes and the like of another mammal (e.g., human) by RT-PCR. V_H and C_H, and V_L and C_L, are ligated together, respectively, using a conveutioual method, and the chimeric H chain DNA and chimeric L chain DNA obtained are inserted into respective appropriate expression vectors [for example, vectors comprising promoters that have transcription activity in CHO cells, COS cells, mouse myeloma cells and the like (c.g., CMV promoter, SV40 promoter and the like)]. The DNAs that eucode the two chains may be inserted into separate vectors, and may be inserted into a siugle vector in tandem. Host cells are transformed with the chimeric H chain and chimeric L chain expression vector(s) obtained. As the host cells, animal cells, for example, Chinese hamster ovary (CHO) cells, monkey-derived COS-7 cells, Vero cells, rat-derived GHS cells and the like, in additiou to the above-described mouse myeloma cells, can be uentioned. For the transformatiou, any method applicable to animal cells can be used, with prefereuce given to electroporation method aud the like. It is possible to isolate a chiueric monoclonal antibody by culturing the host cells in a uedium suitable thereto for a given period, and thereafter recovering the culture supernatant and purifying it in the same manner as described above. Alternatively, it is also possible to obtain a chiueric monoclonal antibody easily aud in large amounts from milk or eggs of transgenic animals which are produced by a conveutional method using germ line cells of an animal such as bovine, goat, or chicken as the host cells, for which a trausgenic techniquc has been established and a know-how of mass propagation as a domestic animal (domestic fowl) has been compiled. Furthermore, it is also possible to obtaiu a chimeric monoclonal autibody in large amounts from the seeds, leaves aud the like of a transgenic plant, produced by using microinjection and electroporation into protoplast, thc particle gun method and Ti-vector mcthod for intact cells and the likc, with cells of a plant such as corn, rice, wheat, soybeau, or tobacco as the host cells, for which a transgenic technique has been established, and which is cultured in large amounts as a major crop.

[0106] When the chimeric monoclonal antibody obtained is digested with papain, Fab is obtained; when the same is digested with pepsin, $F(ab')_2$ is obtained.

[0107] It is also possible to reformat into scFv by ligating DNAs that encode uouse V_H and V_L via a suitable linker, for example, a DNA that encodes a peptide consisting of 1 to 40 amino acids, preferably 3 to 30 amiuo acids, morc preferably 5 to 20 amino acids [e.g., [Ser-(Gly)m]n or [(Gly)m-Ser]n (m is an iuteger from 0 to 10, n is an intcger from 1 to 5) and the like]. Furthermore, it is possible to reformat into a minibody by ligating a DNA that eucodes C_{H3} via a suitable linker thereto, or reformat into a scFv-Fc by ligating a DNA that eucodes C_H full length via a suitable linker thereto. The DNA eucodiug such an autibody moleculc modified (coupled) by genetic engineering can be expressed in a microorganism such as *E. coli* or yeast under the control of a suitable promoter, to produce the antibody molecule in large amounts.

[0108] When DNAs eucoding uouse V_H and V_L are iuserted into the downstream of one promoter in tandem and iutroduced iuto *E. coli*, a dimer named as Fv is formed by monocistronic gene expression. When an appropriate amino acid in the FRs of V_H and V_L is substituted with Cys using molecule modeling, a dimer named as dsFv is formed via the intermolecular disulfide bond betweeu the two chaius.

(2) Humanized Antibody

[0109] As used herein, "a humanized autibody" means an antibody wherein the sequences of all regions present in the variable region, other than the complementality determining region (CDR), [i.e., framcwork regiou (FR) in constant region and variablc region] are derivcd from a humau, and wherein only the sequence of CDR is derived from another mammalian species. The other mammalian species is preferably an animal species, for example, mouse and the like, with which production of hybridomas can be easily performed.

[0110] As examples of the method of preparing a humanized antibody, the methods describcd in U.S. Pat. Nos. 5,225,539, 5,585,089, 5,693,761 and 5,693,762 or partially modified methods therefrom and the like can be mentioned. To be specific, DNAs that eucode V_H and V_L derivcd from a non-human mammalian species (e.g., mouse) are isolated in the same manner as with the above-described chimeric antibody, after which sequenciug is performed by a conventional method using an automated DNA scquencer (e.g., manufactured by Applied Biosystems Company and the like), and the base sequences obtained or deduced amino acid sequcnces thercfrom arc analyzcd using a known antibody sequence database [for example, Kabat database (see Kabat et al., "Sequences of Proteins of Immunological Interest", edited by NIH, US Department of Health and Human Services, Public Health Service, 5th edition, 1991) and the like] to determine the CDR and FR of the two chains. A base sequence wherein the CDR encoding region of a base sequence that encodes the L chain and H chain of a human antibody having an FR sequence similar to the determined FR sequeuce [e.g., human κ type L chain subgroup I and human H chain subgroup II or III (see Kabat et al., 1991 (supra))] is substituted with the determined base sequence that eucodes the CDR of another animal species, is designed, and the base sequence is divided into fragments of about 20 to 40 bases, and a sequence complementary to the base sequence is divided into fragments of about 20 to 40 bases so that thcy alternatively overlap with the aforcmentioned fragments. It is possible to construct DNAs that encode V_H and V_L having human-derived FR and a CDR derived from another mammaliau species by syuthesiziug individual fragments using a DNA synthesizer, and hybridizing and ligatiug them in accordance with conventional methods. In order to transfer a CDR derived from another mammalian species into human-dcrivcd V_H and V_L more quickly and more efficiently, it is preferable to use PCR-based site directed mutagenesis. As examples of such a method, the sequential CDR grafting method described in Japauese Patent Unexamined Publication No. HEI-5-227970 and the like can be mentioned. It is possible to obtain cells or transgenic animal/plant that produces a humanized antibody by ligating the thus-obtainecd DNAs that encode V_H and V_L to DNAs that encode human-derived C_H and C_L, respectively, in the same manner as with the above-described chimeric antibody, and introducing the ligated product into suitable host cells.

[0111] A humanized antibody, like a chimeric antibody, can be modified to scFv, scFv-Fc, minibody, dsFv, Fv aud the like by usiug genetic eugineeriug techniques; and they can be produced in a microorganism such as *E. coli* or yeast by using a suitable promoter.

[0112] The technology for preparing a humanized antibody can also be applied to, for example, preparing a monoclonal antibody that can be preferably administered to another animal species for which no hybridoma production technology has been established. For example, animals widely propagated as domestic animals (domestic fowls) such as bovine, swine, sheep, goat, and chicken, and pet animals such as dogs and cats, and the like can be mentioned as the subject animal species.

(3) Preparation of Fully Human Antibody Using Human Antibody-Producing Animal

[0113] Provided that a functional human Ig gene is introduced into a non-human warm-blooded animal having the endogenous immunoglobulin (Ig) gene knocked out. (KO) therein, and that this animal is immunized with an antigen, a human antibody is produced in place of the antibody derived from the animal. Therefore, provided that an animal such as mice, for which a technique for producing a hybridoma has been established, is used, it is possible to acquire a fully human monoclonal antibody by the same method as the conventional method used to prepare a mouse monoclonal antibody. First, some of the human monoclonal antibodies, that were generated by using a human antibody-producing mouse obtained by crossing a mouse transfected with minigenes of the human Ig H chain and L chain using an ordinary transgenic (Tg) technique with a mouse wherein the endogenous mouse Ig gene has been inactivated using an ordinary KO technique, are already in clinical stage, and to date production of anti-human Ig human antibody (HAHA) has not been reported.

[0114] Later, Abgenix Inc. [trade name: XenoMouse (see *Nat. Genet.*, 15: 146-156, 1997; U.S. Pat. No. 5,939,598 and the like)] and Medarex Inc. [trade name: Hu-Mab Mouse (see *Nat. Biotechnol.*, 14: 845-851, 1996; U.S. Pat. No. 5,545,806 and the like)] established Tg mice transfected with even a larger human Ig gene using a yeast artificial chromosome (YAC) vector, thus enabling the production of human antibodies of richer repertoire. However, because the human Ig gene, for example, in the case of the H chain, exhibits its diversity as the VDJ exon, which is a variable combination of about 80 kinds of V fragments, about 30 kinds of D fragments and 6 kinds of J fragments, encodes the antigen binding site, the full length thereof is as large as about 1.5 Mb (14th chromosome) for the H chain, about 2 Mb (2nd chromosome) for the κL chain, and about 1 Mb (22nd chromosome) for the λL chain. To reproduce the diverse antibody repertoire in human in another animal species, it is desirable to introduce the full length of each Ig gene. However, a DNA that is insertable into a conventional transfection vector (plasmid, cosmid, BAC, YAC and the like) is normally several kb to several hundred kb in length, and it has been difficult to introduce the full length of Ig genes by the conventional technique for establishing a transgenic animal, which comprises inserting a cloned DNA into a fertilized egg.

[0115] Tomizuka et al. (*Nat. Genet.*, 16: 133-143, 1997) prepared a mouse having the full-length human Ig gene by introducing a natural fragment of a human chromosome harboring the Ig gene (hCF) into a mouse [transchromosomic (TC) mouse]. That is, first, a human-mouse hybrid cell having human chromosomes in which the 14th chromosome comprising the H chain gene and the 2nd chromosome comprising the κL chain gene, both labeled with, for example, a drug-resistance marker and the like, is treated with a spindle formation inhibitor (e.g., colcemid) for about 48 hours to prepare a microcell wherein one to several chromosomes or fragments thereof are enveloped in nuclear membrane, and the chromosomes are introduced into a mouse ES cell by the micronuclear fusion method. A hybrid ES cell retaining the chromosomes having the human Ig gene or fragments thereof is selected using a medium containing a drug, and the cell is microinjected into a mouse embryo in the same manner as with the preparation of an ordinary KO mouse. A germ line chimera is selected among the chimeric mice obtained, with coat color as the index, and the like, to establish a TC mouse strain carrying the human 14th chromosome fragment ($TC(hCF_{14})$) and a TC mouse strain carrying the human 2nd chromosome fragment ($TC(hCF_2)$). After establishing mouse strains wherein the endogenous H chain gene and κL chain gene are knocked out, respectively [KO(IgH) and KO(IgK)] by a conventional method, it is possible to establish a mouse strain having all the four kinds of gene modifications (double TC/KO) by repeating the crossing of these four strains.

[0116] Provided that the same method as that for producing an ordinary mouse monoclonal antibody is applied to a double TC/KO mouse established as described above, it is possible to obtain an antigen-specific human monoclonal antibody-producing hybridoma. However, there is the drawback of a lower efficiency to obtain hybridomas than that with the ordinary mouse, because hCF_2 containing the κL chain gene is unstable in the mouse cells.

[0117] On the other hand, because the aforementioned Hu-Mab mouse has a structure wherein the variable region cluster are doubled although it has about 50% of the κL chain gene, it exhibits a κ chain diversity equivalent to that with full length (on the other hand, HuMab mouse exhibits a low H chain diversity and inadequate response to antigen because it carries only about 10% of the H chain gene). And the κ chain is stably retained in the mouse cells because it is inserted in mouse chromosome via a YAC vector (IgκYAC). Making use of this advantage, it is possible to get the efficiency for obtaining hybridomas and affinity to antigen affinity of antibody that are equivalent to those with the ordinary mouse, by crossing a $TC(hCF_{14})$ mouse with a Hu-Mab mouse to establish a mouse that stably retains both hCF14 and IgκYAC (trade name: KM mouse).

[0118] Furthermore, it is also possible to establish a human antibody-producing animal in which the λL chain gene is further transfected to reconstruct the diverse human antibody repertoire more completely. Such an animal can also be obtained by producing a TC mouse in which the human 22nd chromosome or a fragment thereof harboring the λL chain gene is introduced in the same manner as described above[TC(hCF22)], and crossing the mouse with the above-described double TC/KO mouse or KM mouse, or can also be obtained by, for example, constructing a human artificial chromosome (HAC) comprising both the H chain locus and the λL chain locus, and introducing it into a mouse cell (*Nat. Biotechnol.*, 18: 1086-1090, 2000).

[0119] An antibody used in the present invention is desirably a monoclonal antibody because it must be a non-neutralizing antibody. However, it is possible in principle to obtain a non-neutralizing antibody even if it is a polyclonal

antibody because an antibody that binds to the specific site of the target antigen can be obtained using a small hapten molecule as the antigen. When the antibody used in the present invention is a polyclonal antibody, it is not necessary to use hybridomas; therefore, provided that a human antibody-producing animal is produced in the same manner as described above using an animal species for which no technique for preparing a hybridoma has been established but a transgenic technique has been established, preferably an ungulate such as bovine, it is also possible to produce a human antibody in larger amounts at low costs (see, for example, *Nat. Biotechnol.*, 20: 889-894, 2002). The human polyclonal antibody thus obtained can be purified by collecting blood, ascites fluid, milk, egg and the like, preferably milk or egg, of the human antibody-producing animal, in combination with the same purification techniques as described above.

(4) Preparation of Fully Human Antibody Using Phage Display Human Antibody Library

[0120] Another approach to produce a fully human antibody is a method using phage display. This method sometimes encounters cases in which a mutation due to PCR is introduced into a site other than CDRs; for this reason, a few reports of cases of HAHA production in clinical stage are available. On the other hand, however, the method has advantages such as no risk of cross-species viral infection derived from the host animal and the indefinite specificity of the antibody (antibodies against forbidden clone, sugar chain and the like can also be easily prepared).

[0121] The method of preparing a phage display human antibody library include, but are not limited to, for example, the methods described below.

[0122] Although a phage used is not subject to limitation, filamentous phage (Ff bacteriophage) is normally preferably used. As the method of presenting a foreign protein on the phage surface, a method comprising expressing and presenting the foreign protein as a fusion protein with any of the coat proteins g3p, and g6p to g9p on the coat protein can be mentioned; and a method comprising fusing the foreign protein to the N-terminal side of g3p or g8p is often used. As the phage display vector, besides 1) one in which the foreign gene is introduced in the form of fusion gene with the coat protein gene of the phage genome, to allow all the coat proteins presented on the phage surface to be presented as a fusion protein with the foreign protein, 2) one in which the gene encoding the fusion protein is inserted separately from the wild-type coat protein gene to allow the fusion protein and the wild-type coat protein to be expressed simultaneously, and 3) an *E. coli* having a phagemid vector harboring the gene that encodes the fusion protein is infected with a helper phage having the wild-type coat protein gene to produce phage particles that express the fusion protein and the wild-type coat protein simultaneously, and the like can be mentioned.

[0123] However, a phage display vector of the type 2) or 3) is used for the preparation of an antibody library, because in the case of 1), the capability of infection is lost when a large foreign protein is fused.

[0124] As a specific vector, those described by Holt et al. (*Curr. Opin. Biotechnol.*, 11: 445-449, 2000) can be mentioned as examples. For example, pCES1 (see *J. Biol. Chem.*, 274: 18218-18230, 1999) is an Fab-expressing phagemid vector wherein a DNA encoding the κL chain constant region allocated to downstream of the g3p signal peptide, and a DNA encoding CH3, His-tag, c-myc tag, and the amber stop codon (TAG) followed by the g3p coding sequence, allocated to downstream of the g3p signal peptide, are arranged under the control of one lactose promoter. When this is introduced to an *E. coli* having an amber mutation, Fab is presented onto the g3p coat protein, but when it is expressed in the HB2151 strain and the like, which do not have an amber mutation, a soluble Fab antibody is produced. And as the scFv-expressing phagemid vector, for example, pHEN1 (*J. Mol. Biol.*, 222: 581-597, 1991) and the like are used.

[0125] Meanwhile as examples of the helper phage, M13-KO7, VCSM13 and the like can be mentioned.

[0126] And as another phage display vector, a vector that is designed as a DNA sequence comprising the cysteine-encoding codon is linked to each of the 3' end of the antibody gene and the 5' end of the coat protein gene to express the two genes simultaneously and separately (not in the form of a fusion protein), and to present the antibody onto the coat protein on the phage surface via S—S bonds between the introduced cysteine residues (CysDisplay™ technology of Morphosys Company) and the like, can be mentioned.

[0127] As the kind of human antibody library, a naive/non-immunized library, a synthetic library, an immunized library and the like can be mentioned.

[0128] The naive/non-immunized library is a library obtained by acquiring the V_H and V_L genes retained by a normal human by RT-PCR, and randomly cloning them into the above-described phage display vector. Normally, mRNA derived from lymphocytes of peripheral blood, bone marrow, tonsil and the like of a normal human, and the like are used as the template. A library prepared by selectively amplifying IgM-derived mRNA in which a class switch due to antigen sensitization is not undergoing, to avoid V gene biases such as clinical history, is particularly called a naive library. Representatively, the library of Cambridge Antibody Technology (see *J. Mol. Biol.*, 222: 581-597, 1991; *Nat. Biotechnol.*, 14: 309-314, 1996), the library of Medical Research Council (see *Annu. Rev. Immunol.*, 12: 433-455, 1994), the library of Dyax Corp. (see *J. Biol. Chem.*, 1999 (supra); *Proc. Natl. Acad. Sci. USA*, 14: 7969-7974, 2000) and the like can be mentioned.

[0129] A synthetic library is obtained by selecting a functional particular antibody gene in human B cells, and substituting a portion of antigen-binding region in a V gene fragment, for example, CDR3 and the like, with DNAs encoding a random amino acid sequence of appropriate length, to construct a library. It is recognized to be excellent in antibody expression efficiency and stability because the library can be constructed with the combination of the V_H and V_L genes, which produce functional scFv and Fab, since the beginning. Representatively, the HuCAL library of Morphosys AG (see *J. Mol. Biol.*, 296: 57-86, 2000), the library of BioInvent (see *Nat. Biotechnol.*, 18: 852, 2000), the library of Crucell (see *Proc. Natl. Acad. Sci. USA*, 92: 3938, 1995; *J. Immunol. Methods*, 272: 219-233, 2003) and the like can be mentioned.

[0130] An immunized library is a library obtained by preparing an mRNA from lymphocytes collected from a

human such as a patient with cancer, autoimmune disease, infections disease and the like or a recipient of vaccination, having an elevated blood antibody titer against the target antigen, or from human lymphocytes and the like which are artificially immunized with the target antigen by the above-described in vitro immunization method, in the same manner as with the above-described naive/non-immunized library, and amplifying the V_H and V_L genes by RT-PCR, to construct a library. It is possible to obtain the desired antibody even from such libraries of relatively small size because the desired antibody gene is contained in the library already at the beginning.

[0131] The wider the diversity of the library is, the better; actually, however, an appropriate library size is about 10^8 to 10^{11} clones, taking into consideration of the number of phages handlable in the following panning operation (10^{11} to 10^{13} phages) and the number of phages necessary to isolate and amplify clones in ordinary panning (100 to 1,000 phages/clone), it is possible to screen for an antibody normally having a Kd value on the order of 10^{-9} with a library of about 10^8 clones.

[0132] The process for selecting an antibody against the target antigen by the phage display method is referred to as panning. To be specific, for example, a phage presenting an antigen-specific antibody is concentrated by repeating a series of operations of bringing an antigen-immobilized carrier and a phage library into contact with each other, washing out the unbound phage, thereafter eluting the bound phage from the carrier, and infecting the phage to *E. coli* to proliferate it, about 3 to 5 times. As the carrier for immobilizing the antigen, various carriers used in ordinary antigen-antibody reactions or affinity chromatography, for example, insoluble polysaccharides such as agarose, dextran, and cellulose, synthetic resins such as polystyrene, polyacrylamide, and silicon, or microplates, tubes, membranes, columns, beads and the like comprising glass, metal and the like, and surface plasmon resonance (SPR) sensor chips, and the like can be mentioned. For the antigen immobilization, physical adsorption may be used, and a method using a chemical bond used to insolubilize and immobilize a protein or enzyme and the like is also acceptable. For example, a biotin-(strept)avidin system and the like are preferably used. When the endogenous ligand, that is a target antigen, is a small molecule such as a peptide, it is necessary to pay special attention to prevent masking of the portion used as the epitope by conjugating with the carrier. For washing the unbound phage, a blocking solution such as BSA solution (once or twice), a PBS containing a surfactant such as Tween (3 to 5 times) and the like can be used. There is also a report mentioning that the use of citrate buffer (pH 5) and the like is preferable for the washing. For elution of the specific phage, an acid (e.g., 0.1 M hydrochloric acid and the like) is normally used; cleavage with a specific protease (for example, a gene sequence that encodes the trypsin cleavage site can be introduced into the linkage site between the antibody gene and the coat protein gene. In this case, *E. coli* infection and proliferation are possible even if all the coat protein is expressed in the form of a fusion protein because the wild-type coat protein is presented on the surface of the eluted phage), competitive elution with a soluble antigen, or elution by reduction of S—S bond (for example, in the aforementioned CysDisplay™, the antigen-specific phage can be recovered by dissociating the antibody and the coat protein by using a suitable reducing agent after performing panning) is also possible. When elution has been performed with an acid, the eluate is neutralized with Tris and the like, and the eluted phage is then infected to *E. coli*, which is cultured; after which the phage is recovered by a conventional method.

[0133] After the phage presenting the antigen-specific antibody is concentrated by panning, the phage is infected to *E. coli* and the cells are sown onto a plate to perform cell cloning. The phage is again collected from the each clone, and the antigen binding activity is confirmed by the above-described antibody titer assay (e.g., ELISA, RIA, FIA and the like) or a measurement utilizing FACS or SPR.

[0134] Isolation and purification of the antibody from the selected phage clone that presents the antigen-specific antibody can be performed by, for example, when using a vector incorporating an amber stop codon at the linker site of the antibody gene and the coat protein gene as the phage display vector, infecting the phage to an *E. coli* that does not have amber mutation (e.g., HB2151 strain) to produce and secrete soluble antibody molecules in periplasm or the medium, lysing the cell wall with lysozyme and the like, collecting the extracellular fraction, and purifying using the same purification technique as described above. Provided that the His-tag or c-myc tag has been introduced in advance, the antibody can easily be purified by using IMAC, an anti-c-myc antibody column and the like. When cleavage with a specific protease is utilized in panning, the antibody molecule is separated from the phage surface by an action with the protease, so that the desired antibody can be purified by performing the same purification operation as above mentioned.

[0135] Confirmation of the thus-obtained antibody to be a non-neutralizing antibody can be achieved in the same manner as with the above-described heterologous monoclonal antibody.

[0136] The technology for producing a fully human antibody using a human antibody-producing animal and a phage display human antibody library can also be applied to the production of a monoclonal antibody derived from another animal species. For example, animals widely propagated as domestic animals (domestic fowls) such as bovine, swine, sheep, goat, and chicken, and pet animals such as dogs and cats, and the like can be mentioned as the subject animal species. In non-human animals, the utilization of an immunized library is more effective because there are fewer ethical problems concerning artificial immunization with the target antigen.

[0137] The non-neutralizing antibody (preferably a monoclonal antibody) against an endogenous ligand, obtained as described above, can be used as an agent for improving the blood stability of the endogenous ligand, after being mixed with a pharmacologically acceptable carrier into a pharmaceutical composition, as required.

[0138] As the pharmacologically acceptable carrier here, various organic or inorganic carrier substances conventionally used as materials to prepare medicine can be used, which is compounded into as excipient, solvent (dispersing agent), solubilizer, suspending agent, stabilizer, isotonizing agent, buffer, pH adjusting agent, soothing agent and the like. Where necessary, preparation additives such as preservatives, antioxidants and the like can also be used.

[0139] As examples of preferable excipieuts, lactose, sucrose, D-mannitol, D-sorbitol, starch, α-starch, dextrin, crystalliue cellnlose, low-substituted hydroxypropylcellulose, carboxymethylcellulose sodium, gum arabic, pullulan, light silicic anhydride, synthetic aluminum silicate, magnesium metasilicate aluminate and the like can be mentioned.

[0140] As examples of preferable solvents, water for injection, physiological salinc, Ringer's solution, alcohol, propylene glycol, polyethylene glycol, sesame oil, corn oil, olive oil, cottonseed oil and the like can be mentioned.

[0141] As examples of preferable solubilizers, polyethyleue glycol, propylene glycol, D-mannitol, trehalose, benzyl benzoate, ethanol, trisaminomethane, cholesterol, triethanolamine, sodium carbonate, sodium citrate, sodium salicylate, sodium acctatc aud the like can be meutioned.

[0142] As examples of preferable suspending agents, surfactants such as stearyltriethanolamine, sodium lanryl sulfate, laurylaminopropionic acid, lecithin, benzalkonium chloride, benzethonium chloride, and glyceryl monostearate; hydrophilic polymers such as polyvinyl alcohol, polyvinylpyrrolidone, carboxymethylcellulose sodium, methylcellulose, hydroxymethylcellulose, hydroxyethylcellulose, and hydroxypropylcellulose; polysorbates, polyoxyethylene hydrogenated castor oil and the likc can bc mentioned.

[0143] As examples of preferable stabilizers, human serum albnmin (HSA), sodinm pyrosulfite, Rongalit, sodium metahydrogensulfite and the like can be mentioned.

[0144] As examples of preferable isotonizing agents, sodium chloride, glycerin, D-mannitol, D-sorbitol, glncose and the like can be mentioned.

[0145] As examples of preferable buffers, buffer solutions such as of phosphates, acetates, carbonates and citrates, and thc like can be mentioned.

[0146] As cxamples of preferable pH adjusting agent, acid or base such as hydrochloride, sodium hydroxide and the like can be mentioned.

[0147] As examples of preferable soothiug ageuts, benzyl alcohol and the like can be mentioned.

[0148] As examples of preferable preservatives, p-hydroxybenzoates, chlorobutanol, beuzyl alcohol, phenethyl alcohol, dehydroacetic acid, sorbic acid and the like can be mentioned.

[0149] As examples of preferable antioxidants, sulfites, ascorbic acid salts and the like can be mentioned.

[0150] As examples of dosage forms for the aforementioned pharmaceutical composition, injection type preparations such as injections (e.g., subcutaneous injections, intravenous injections, intramuscular injections, intraperitoueal injections, intraarterial iujectious and the like), drip infusions and the like can be mentioned.

[0151] Thesc pharmaccutical compositions can be produced by a method in common use in the field of drug formulation technology, for example, methods described in the Japanese Pharmacopoeia and the like. Specific methods of preparing prcparations are described in detail below. The antibody content in the pharmaceutical composition varies depeuding on dosage form, antibody dose and the like, and is, for cxample, about 0.1% to 100% by wcight.

[0152] For example, an injection is produced by dissolving, snspending or emnlsifying antibody, along with a dispersing agent (e.g., polysorbate 80, polyoxyethylene hydrogenated castor oil 60, polyethylene glycol, carboxymethylcellulose, sodium alginate and the like), a preservative (e.g., methylparaben, propylparaben, benzyl alcohol, chlorobutanol, phenol and the like), an isotonizing agent (e.g., sodium chloride, glycerin, D-mannitol, D-sorbitol, glncose and the like) and the like, in an aqneons solvent (e.g., distilled water, physiological saline, Ringer's solntion and the like) or an oily solvent (e.g., vegetable oils such as olive oil, sesame oil, cottonseed oil and corn oil, propylene glycol and the like). 1f desired, additives snch as a solubilizer (e.g., sodium salicylate, sodium acetate and the like), a stabilizer (e.g., hnman serum albnmin and the like), and a soothing agent (e.g., benzyl alcohol and the like) may be used. The injection is subjected, for example, to a sterilization treatment as necessary, such as sterilization by filtration nsing a membrane filter etc., aud usually filled in a snitable container such as ampoule etc.

[0153] The injection can also be used as a fresh supply obtained by dissolving (dispersing) a powder prepared by treating the above-dcscribed liquid by vacuum drying and the likc. As cxamples of the vacuum drying mcthod, lyophilization, a method using the Speedback Concentrator (SAVANT Company), and the like can be mentioned. When performing lyophlilization, it is preferable to lyophilize the sample, cooled below −10° C., using a flask in the laboratory or a tray or vial in industrial settings. When the Speedback Concentrator is used, lyophilization is performed at abont 0 to 30° C. under a vacuum of about 20 mm/Hg or less, preferably abont 10 mmHg or less. It is preferable to add a bnffering agent such as a phosphate to the liquid to be dried, to obtain a pH of abont 3 to 10. The powder preparation obtained by lyophilizatiou, as a long-stable preparation, can be prepared freshly as an injection by dissolving in water for injection, saline, Riuger's solution and the like, or by dispersiug in olive oil, sesame oil, cottonseed oil, corn oil, propyleneglycol and the like before use.

[0154] Because the agent for improviug the blood stability of the present invention, which is obtained as above, is safe and less toxic, it can be administered to, for example, mammals (for example, hnmans, rats, rabbits, sheep, swine, cattle, cats, dogs, monkeys and the like) subcutaneously, intravenonsly, intramuscularly, intraperitoneally, intraarterially or intraventricularly.

[0155] The dose of the agent for improving the blood stability of thc present inventiou is not subject to limitation, as long as it is an amount enabling an increase in the blood concentration of an endogenons ligand in the recipient mammal to a range effective for the treatment or prophylaxis for a disease the animal has contracted or can contract, and/or enabling an cxtension of the blood half-life of the endogenons ligand to an extent effective for the treatment or prophylaxis for the disease. For example, when the agent for improving the blood stability of thc present invention is snbcutaneously administered to a human for the purpose of improving the blood stability of endogenous GLP-1, the minimum required concentration in blood of GLP-1 and a functional fragment thereof is estimated to be about 100 pM in total. If we assnme that a 10- to 100-fold amount of anti-GLP-1 antibody is necessary to form an immune complex with all blood GLP-1, a requircd blood GLP-1 concen-

tration can be achieved by administering about 1 to 10 nM or, in terms of weight, about 10 to 100 μg/kg body weight, per dosing. This amount can be administered at intervals of, for example, one to several weeks.

[0156] However, the dose also varies depending on the normal blood concentration and blood half-life of the target endogenous ligand, stability of the antibody molecule, tissue transfer of the ligand-antibody complex, antigen affinity of the antibody, neutralization activity of the antibody, target disease, severity, in addition, animal species, age, route of administration and the like. Therefore, the general range of the dose of the agent for improving the blood stability of the present invention is even wider; for example, about 0.1 μg to 10 mg/kg, preferably about 1 μg to 1 mg/kg, per dosing can be used.

[0157] Preferably, when the agent for improving the blood stability of the present invention is administered to a mammal, the blood half-life of the complex of the endogenous ligand and the antibody is extended more than about 2 times, more preferably more than about 10 times, particularly preferably more than about 50 times, compared with that in the case of the endogenous ligand alone (i.e., free form). Such an elongation effect results in a blood concentration of the endogenous ligand with administration of the agent for improving the blood stability of the present invention to a mammal, more than about 2 times, more preferably more than about 5 times, particularly preferably more than about 10 times, higher than the blood concentration obtained without administering the preparation. As mentioned herein, a blood concentration is measured at an optionally chosen time between a dosing and the subsequent dosing.

[0158] Because the agent for improving the blood stability of the present invention can stabilize an endogenous ligand by the action of the antibody which is the active ingredient of the preparation, as described above, and also because the antibody substantially does not neutralize the antigen endogenous ligand, the agent of the present invention can be used as a prophylactic or therapeutic agent for a disease for which enhancing the receptor activity-regulatory action of the ligand by increasing the blood concentration of the endogenous ligand and/or extending the blood half-life is prophylactically or therapeutically effective.

[0159] Examples of such diseases include, but are not limited to, metabolic disease, bone and joint disease, cardiovascular disease, cranial/nerve disease, infectious disease, cancer, blood disorder, urologic disease, infertility/erectile dysfunction, deficient growth and immunodeficiency and the like.

[0160] Describing in more detail the target disease in relation to association with individual endogenous ligands serving as target antigens, examples of the metabolic disease include diabetes (target ligand: GLP-1, calcitonin, PACAP, VIP, insulin, beta-cellulin, beta-cellulin-δ4 and the like), obesity (target ligand: leptin, adiponectin, GPR7/GPR8 ligand (NPW), MSH and the like), anorexia (target ligand: ghrelin and the like) and the like; examples of the bone and joint disease include osteoporosis (target ligand: calcitonin, PTH and the like) and the like; examples of the cardiovascular disease include ischemic heart diseases (myocardial infarction, angina pectoris) (target ligand: ghrelin, adrenomedullin and the like), hypertension (target ligand: ANP, BNP, CNP and the like) and the like; examples of the cranial nerve disease include ischemic cranial neuropathy (target ligand: ghrelin, VIP and the like), Alzheimer's disease (target ligand: LH-RH and the like) and the like; examples of the infectious disease include viral infectious disease (target ligand: interferon, GM-CSF and the like) and the like; examples of the cancer include prostate cancer, breast cancer (target ligand: LH-RH and the like), primary carcinoma or metastatic carcinoma of various organs (target ligand: metastin, interferon, TNF, IL-2, M-CSF, GM-CSF and the like) and the like; examples of the blood disorder include anemia (target ligand: EPO, GM-CSF and the like) and the like; examples of the urologic disease include dysuria (target ligand: ANP, BNP, CNP and the like) and the like; examples of the infertility/erectile dysfunction include infertility (target ligand: metastin and the like), erectile dysfunction (target ligand: VIP and the like) and the like; examples of the deficient growth include growth hormone deficient short statue (hyperphysical dwarf), Turner's syndrome, Prader-Willi syndrome (target ligand: growth hormone) and the like; examples of the immunodeficiency include HIV infection, after immunosuppressant administration at the time of transplantation or treatment for autoimmune disease (target ligand: GM-CSF and the like) and the like.

[0161] When amino acid etc. are to be indicated with abbreviations in this specification, the abbreviations are adopted from IUPAC-IUB Commission on Biochemical Nomenclature or those commonly used in the art. For example, the following abbreviations are used. When an optical isomer is capable of existing with respect to the amino acids, the L-form is represented unless otherwise specified.

[0162] PAM: Phenylacetamidomethyl

[0163] BHA: Benzhydrylamine

[0164] Boc: t-Butyloxycarbonyl

[0165] Cl-Z: 2-Chloro-benzyloxycarbonyl

[0166] Br-Z: 2-Bromo-benzyloxycarbonyl

[0167] Bzl: Benzyl

[0168] OBzl: Benzyl ester

[0169] Tos: p-Toluenesulfonyl

[0170] HOBt: 1-Benzotriazole

[0171] DCC: N,N'-Dichlorohexylcarbodiimide

[0172] Gly: Glycine

[0173] Ala: Alanine

[0174] Val: Valine

[0175] Leu: Leucine

[0176] Ile: Isoleucine

[0177] Ser: Serine

[0178] Thr: Threonine

[0179] Cys: Cysteine

[0180] Met: Methionine

[0181] Glu: Glutamic acid

[0182] Asp: Aspartic acid

[0183] Lys: Lysine

[0184] Arg: Arginine

[0185] His: Histidine

[0186] Phe: Phenylalanine

[0187] Tyr: Tyrosine

[0188] Trp: Tryptophau

[0189] Pro: Proline

[0190] Asu: Asparagine

[0191] Gln: Glutamine

[0192] The present invention is explained in detail in the following by referring to Examples, which are mere exemplifications and not to be construed as limiting the scope of the present invention.

[0193] The anti-PACAP antibody-producing hybridoma obtained in the Examples below has been deposited with the Fermentation Research Institute, the Agency of Industrial Science and Technology, the Ministry of International Trade and Iudustry, Japan (then). [now the International Patent Organism Depositary (IPOD), National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1, Higashi, Tsukuba, Ibaraki, 305-8566 Japan)] under the following accession numbers since Mar. 16, 1990:

[0194] hybridoma cell

[0195] PA-1N:FERM BP-2811

[0196] PA-3N:FERM BP-2812

[0197] PA-5N:FERM BP-2813

[0198] PA-6N:FERM BP-2814

[0199] PA-2C:FERM BP-2815

[0200] PA-1C:FERM BP-2816

[0201] In the Examples below, antibodies obtained from each hybridoma cell are denoted with an "a" after the name of the cell (for example, antibody obtained from PA-1N cell is PA-1Na).

[0202] In addition, hybridoma (GLIT2-329(1)24) producing auti-GLP-1 non-neutralizing antibody GLIT2-329(1)24 was accepted as of Mar. 17, 2005 by the IPOD (mentioned above) under the accession No. of FERM ABP-10297.

REFERENCE EXAMPLE 1

(1) Synthesis of $PACAP38NH_2$

[0203] $PACAP38NH_2$ (SEQ ID NO:1) was synthesized by usiug 1.04 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) aud a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

[0204] A starting amino acid, Boc-Lys(Cl-Z), was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequeuce of $PACAP38NH_2$:

[0205] Boc-Asu, Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Gln, Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met, Boc Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc Ile, and Boc-His(Tos). After the additional condensation by the same amino acid derivatives activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 2.42 g of a protected $PACAP38NH_2$ resin.

[0206] 0.51 g of the resultiug protected $PACAP38NH_2$ resin was treated with 5 ml of hydrogen fluoride in the presence of 0.6 g of p-cresol at 0° C. for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue washed twice with 5 ml of ethyl ether, and then extracted with 6 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 column (2×90 cm), and eluted with 50% acetic acid. The main fractions were collected, followed by removal by distillation under reduced pressure. Then, the residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was applied on a YMC-ODS AM120 S-50 resiu column (1.6×7 cm) and eluted by a linear concentration gradient of between 0.1% aqueous trifluoroacetic acid and 50% acetonitrile (containing 0.1% trifluoroacetic acid).

[0207] The main fractions were combined and lyophilized. Thus, 60 mg of white powder was obtained. This powder was dissolved in 20 ml of 0.05 M aqueous ammonium acetate. The resulting solution was applied on a CM-Cellulofine resin column (1×6 cm) and eluted by a linear concentration gradient of from 0.05 M to 1 M ammonium acetate. The main fractions were combined and applied on a YMC-ODS column (2.6×7 cm) again and eluted by a linear gradient of from 0% to 40% aqueous acetonitrile (containing 0.1% trifluoroacetic acid). The elution fractions containing 28% to 30% acetonitrile were collected and lyophilized. Thus, 21.6 mg of white powder was obtained.

[0208] Analytical values of amino acid composition:

[0209] Asp 2.90(3), Thr 0.84(1), Ser 2.10(3), Glu 2.21(2), Gly 2.00(2), Ala 3.29(3), Val 3.19(3), Met 1.01(1), Ile 0.87(1), Leu 2.09(2), Tyr 3.94(4), Phe 0.92(1), Lys 7.18(7), H is 0.96(1), Arg 4.19(4)

[0210] $(M+H)^+$ by a mass spectrography: 4530

[0211] HPLC elution time: 19.6 minutes

[0212] Column Conditions

[0213] Column: YMC-ODS (AM-301, S-5 120A)

[0214] Eluent: A (0.1% aqueous trifluoroacetic acid)

[0215] B (acetonitrile containing 0.1% trifluoroacetic acid)

[0216] A linear concentratiou gradient elution from the eluent

[0217] A to the eluent B (50 minutes)

[0218] Flow rate: 1.0 ml/minute

(2) Synthesis of $PACAP27NH_2$

[0219] $PACAP27NH_2$ (SEQ ID NO:2) was synthesized by using 1.04 g (0.5 mmole) of a commercially available

p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

[0220] A starting amino acid, Boc-Leu, was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP27NH_2:

[0221] Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tps), Boc-Gln, Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met, Boc-Ser(Bzl), Boc Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc-Ile, and Boc H is (Tos). After the additional condensation by the same amino acid derivatives activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 2.31 g of a protected PACAP27NH_2 resin.

[0222] 0.79 g of the resulting protected PACAP27NH_2 resin was treated with 10 ml of absolute hydrogen fluoride in the presence of 1.2 g of p-cresol at 0° C. for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 column (2×75 cm) for elution with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was applied on a YMC-ODS AM120 S-50 resin column (2.6×7 cm) and eluted by a liner concentration gradient of between 0.1% aqueous trifluoroacetic acid and 50% acetonitrile (containing 0.1% trifluoroacetic acid). The main fractions were combined and was applied onto a YMC-ODS column (2.6×7 cm) again and eluted by a linear concentration gradient of from 15 to 35% aqueons acetonitrile solution (coutaining 0.1% trifluoroacetic acid). The 30 to 32% fractions of acetonitrile were collected and lyophilized. The resulting product was dissolved in 20 ml of 0.05M-aqueous ammonium acetate. The solution was applied onto a CM Cellulofine resin column (1×6 cm) and eluted by a linear concentration gradient of water to 0.33 M-aqueous ammonium acetate. The main fractions (0.18 to 0.22 M) were collected and lyophilized. Thus, 20 mg of white powder was obtained.

[0223] Analytical values for amino acid composition:

[0224] Asp 1.96(2), Thr 0.94(1), Ser 2.57(3), Gln 1.07(1), Gly 0.95(1), Ala 3.00(3), Val 1.96(2), Met 0.88(1), Ile 0.88(1), Leu 1.93(2), Tyr 2.87(3), Phe 0.90(1), Lys 2.91(3), H is 0.94(1), Arg 2.17(2)

[0225] $(M+H)^+$ by a mass spectrography: 3146.7

[0226] HPLC elution time: 21.2 minutes

[0227] Column Conditions

[0228] Column: YMC-ODS (AM-301, S-5 120A)

[0229] Eluent: A (0.1% aqueous trifluoroacetic acid)

[0230] B (acetonitrile containing 0.1% trifluoroacetic acid)

[0231] A linear concentration gradient elution from the elnent A to the eluent B (50 minutes)

[0232] Flow rate: 1.0 ml/minute

(3) Syuthesis of PACAP(14-38)NH_2

[0233] PACAP(14-38)NH_2 (SEQ ID NO:3) was synthesized by nsing 1.04 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

[0234] A starting amino acid, Lys(Cl-Z), was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(14-38)NH_2:

[0235] Boc-Asn, Boc-Lys(C_1-Z), Boc-Val, Boc-Arg-(Tos), Boc-Gln, Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met.

After the additional condensation by the same amino acid derivatives activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 2.00 g of a protected PACAP(14-38)NH_2 resin.

[0236] 0.48 g of the resulting protected PACAP(14-38)NH_2 resin was treated with 5 ml of absolute hydrogen fluoride in the presence of 0.48 g of p-cresol at 0° C. for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 column (2×75 cm) and eluted with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous Trifluoroacetic acid. The resulting solution was applied onto a YMC-ODS AM120 S-50 resin column (2.6×7 cm) and eluted by a liner concentration gradient of between 0.1% aqueous trifluoroacetic acid and 30% acetonitrile (containing 0.1% trifluoroacetic acid). The main fractions were collected and lyophilized. Thus, 20.2 mg of white powder was obtained.

[0237] Analytical values for amino acid composition:

[0238] Asp 1.01(1), Glu 2.01(2), Gly 1.00(1), Ala 3.01(3), Val 2.85(3), Met 0.86(1), Leu 2.08(2), Tyr 1.98(2), Lys 6.37(7), Arg 3.24(3)

[0239] $(M+H)^+$ by a mass spectrography: 3003.6

[0240] HPLC elution time: 13.1 minutes

[0241] Column Conditions

[0242] Column: YMC-ODS (AM-301, S-5 120A)

[0243] Eluent: A (0.1% aqueous trifluoroacetic acid)

[0244] B (acetonitrile containing 0.1% trifluoroacetic acid)

[0245] A linear concentration gradient elntion from the eluent A to the eluent B (25 minutes)

[0246] Flow rate: 1.0 ml/minute

(4) Synthesis of PACAP(1-13)OH

[0247] PACAP(1-13)OH(SEQ ID NO:4) was synthesized by using 0.87 g (0.5 mmole) of a commercially available Boc-Tyr(Br-Z)-OCH_2-PAM resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

[0248] The Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(1-13)OH:

[0249] Boc-Arg(Tos), Boc-Tyr(Br-Z), Boc-Gly, Boc-Ser-(Bzl),

[0250] Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc-Ile, and

[0251] Boc-H is (Tos). After the additional condensation by the same amino acid derivatives activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 1.86 g of a protected PACAP(1-13)OH_2-PAM resin.

[0252] 0.70 g of the resulting protected resin was treated with 10 ml of absolute hydrogen fluoride in the presence of 0.81 g of p-cresol at 0° C. for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueons acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueons acetic acid. The filtrate and the washings were combined, and the combined solution was concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied onto a Sephadex LH-20 column (2×75 cm) for elntion with 50% acetic acid. The main fractions were collected, followed by distillation nnder reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid.

[0253] The resulting solution was applied onto a YMC-ODS AM120 S-50 resin colnmn (2.6×7 cm) and eluted by a liner concentration gradient of between 0.1% aqueous trifluoroacetic acid and 33% acetonitrile (containing 0.1% trifluoroacetic acid). The main fractions were combined and the combined solution was purified again under the same column conditions. The main fractions were collected and lyophilized. Thus, 38 mg of white powder was obtained.

[0254] Analytical values for amino acid composition:

[0255] Asp 2.00(2), Thr 0.93(1), Ser 2.43(3), Glu 1.05(1), Gly 1.00(1), Tyr 1.82(2), Phe 1.02(1), H is 1.31(1), Arg 1.12(1)

[0256] $(M+H)^+$ by a mass spectrography: 1547.5

[0257] HPLC elution time: 12.3 minutes

[0258] Column Conditions

[0259] Colnmn: YMC-ODS (AM-301, S-5 120A)

[0260] Elnent: A (0.1% aqueous trifluoroacetic acid)

[0261] B (acetonitrile containing 0.1% trifluoroacetic acid)

[0262] A linear concentration gradient elntion from the eluent A to the elnent B (25 minutes)

[0263] Flow rate: 1.0 ml/minute

(5) Synthesis of PACAP(4-27)OH

[0264] PACAP(4-27)OH(SEQ ID NO:5) was synthesized by nsing 0.60 g (0.5 mmole) of a commercially available Boc-Leu-OCH_2-PAM resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems Inc.).

[0265] The Boc group on the resin was treated with 50% triflnoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amino acids activated with HOBt/DCC were condensed in turn according to the amino acid sequence of PACAP(4-27)OH:

[0266] Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Gln, Boc-Tyr(Br-Z), Boc-Gly, Boc-Leu, Boc-Ala, Boc-Met, Boc-Ser(Bzl), Boc-Asp(OBzl), Boc-Thr(Bzl), Boc-Phe, Boc-Ile. After the additional condensation by the same amino acid derivatives activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 1.08 g of a protected PACAP(4-27)OCH_2-PAM resin.

[0267] 0.29 g of the resulting protected PACAP(4-27)OCH_2-PAM resin was treated with 5 ml of absolute hydrogen fluoride in the presence of 0.49 g of p-cresol at 0° C. for 60 minntes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueons acetic acid. The filtrate and the washings were combined, and concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 colnmn (2×75 cm) and eluted with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solntion was applied onto a YMC-ODS AM120 S-50 resin column (2.6×7 cm) and eluted by a liner concentration gradient of between 15% acetonitrile (containing 0.1% triflnoroacetic acid) and 50% acetonitrile (containing 0.1% triflnoroacetic acid). The main fractions were collected, and lyophilized to obtain 33 mg of white powder. The powder was dissolved in 20 ml of 0.05M-aqueons ammonium acetate. The solntion was applied onto a CM-Cellurofine resin column (1×6 cm) and eluted by a linear concentration gradient with water to 0.30M-aqueons ammonium acetate. The main fractions (0.18 to 0.22 M) were collected, and lyophilized. Thus, 33 mg of white powder was obtained.

[0268] Analytical values for amino acid composition:

[0269] Asp 1.02(1), Thr 0.98(1), Ser 1.78(2), Glu 1.07(1), Gly 1.02(1), Ala 3.04(3), Val 1.89(2), Met 0.81(1), Ile

0.89(1), Leu 2.00(2), Tyr 2.91(3), Phe 0.90(1), Lys 2.89(3), Arg 2.20(2)

[0270] $(M+H)^+$ by a mass spectrography: 2808.5

[0271] HPLC elution tiue: 14.5 minutes

[0272] Column Conditions

[0273] Columu: YMC-ODS (AM-301, S-5 120A)

[0274] Eluent: A (0.1% aqueous trifluoroacetic acid)

[0275] B (acetonitrile containing 0.1% trifluoroacetic acid) A linear concentration gradient elution from the eluent

[0276] A to the eluent B (35 minutes)

[0277] Flow rate: 1.0 ml/minute

(6) Syuthesis of PACAP(31-38)NH_2

[0278] PACAP(31-38)NH_2 (SEQ 1D NO:6) was synthesized by using 0.98 g (0.5 mmole) of a commercially available p-methyl BHA resin (Applied Biosystems Inc.) and a peptide synthesizer (Model 430A, Applied Biosystems 1uc.).

[0279] A starting amino acid, Boc-Lys(Cl-Z), was activated with HOBt/DCC and then condensed to the resin. Thereafter, the Boc group on the resin was treated with 50% trifluoroacetic acid/methylene chloride to deprotect the amino group. To this free amino group, the following protected amiuo acids activated with HOBt/DCC were condensed in turn according to thc amino acid sequeucc of PACAP(31-38)NH_2:

[0280] Boc-Asn, Boc-Lys(Cl-Z), Boc-Val, Boc-Arg(Tos), Boc-Glu, Boc-Tyr (Br-Z). After the additional condensation by the same amino acid derivatives activated by DCC or HOBt/DCC, the unreacted amino groups were acetylated with acetic anhydride to obtain 2.00 g of a protected PACAP(31-38)NH_2 rcsiu.

[0281] 0.43 g of the resultiug protected PACAP(31-38)NH_2 resin was treated with 5 ml of absolute hydrogen fluoride in the presence of 0.6 g of p-cresol at 0° C. for 60 minutes, followed by removal of excess hydrogen fluoride by distillation under reduced pressure. The residue washed twice with 5 ml of ethyl ether, and then extracted with 5 ml of 50% aqueous acetic acid. The insoluble material was removed by filtration and washed with 5 ml of 50% aqueous acetic acid. The filtrate and the washings wcre combincd, and concentrated to 2 to 3 ml under reduced pressure. The concentrated solution was applied on a Sephadex LH-20 column (2×75 cm) and eluted with 50% acetic acid. The main fractions were collected, followed by distillation under reduced pressure. The residue was dissolved in 100 ml of 0.1% aqueous trifluoroacetic acid. The resulting solution was applied onto a YMC-ODS AM120 S-50 resin column (2.6×7 cm) and eluted by a liner concentration gradient of 0.1% aqueous trifluoroacetic acid and 33% acetouitrile (containiug 0.1% trifluoroacetic acid). The main fractions were collected, and lyophilized. Thus, 45 mg of white powder was obtaiued.

[0282] Analytical values for amino acid composition:

[0283] Asp 1.02(1), Glu 1.05(1), Val 1.00(1), Tyr 0.90(1), Lys 2.98(3), Arg 1.12(1)

[0284] $(M+H)^+$ by a mass spectrography: 1062.7

[0285] HPLC elution time: 11.6 minutes

[0286] Column Conditions

[0287] Column: YMC-ODS (AM-301, S-5 120A)

[0288] Eluent: A (0.1% aqueous trifluoroacetic acid)

[0289] B (acetonitrile containing 0.1% trifluoroacetic acid)

[0290] A linear concentration gradient elutiou from the eluent

[0291] A to the eluent mixture [A:B(4:1)] (20 minutes)

[0292] Flow rate: 1.0 ml/minute

EXAMPLE 1

(1) Preparation of Immunogen Containing PACAP38NH_2

[0293] A complex comprising PACAP38NH_2 obtained in Reference Example 1(1) described above and bovine thyroglobulin (BTG) was prepared, and it was used as an immunogen. Namely, 2.8 mg of PACAP38NH_2 and 8.4 mg of BTG were dissolved in 1 ml of 0.1 M phosphate buffer (pH 6.9), and glutaraldehyde was added thereto to a final conccutration of 0.04%, followcd by reaction at room temperature for 2 hours. After the reaction, the resulting product was dialyzed against saline at 4° C. for 2 days.

(2) Immunization of PACAP38NH_2-BTG Complex

[0294] The female BALB/C mice (6 to 8 weeks old) were subcutaneously immunized with 80 μg/mouse of the immunogen PACAP38NH_2-BTG complex, obtained in above (1), together with Freund's complete adjuvant. Then, the mice were additionally immunized with the same amount of the immunogen, together with Freund's incomplete adjuvant, 2 to 3 times at 4-week iutervals.

EXAMPLE 2

(1) Preparation of Horseradish Peroxidase (HRP) Labeled PACAP38NH_2

[0295] A labelcd PACAP38NH_2 for the enzyme immunoassay (EIA) was prepared by crosslinking PACAP38NH_2 obtained in Reference Example 1(1) and HRP. Namely, 180 nmoles of PACAP38NH_2 was dissolved in 500 μl of 0.1 M phosphate buffer (pH 6.8), and 50 μl of a DMF solutiou containing 450 nmoles of GMBS was mixed therewith, followed by reaction at room temperature for 30 minutes. After the reactiou, the resulting product was fractionated on a Sephadex G-15 column. Thus, 100 nmoles of a maleimide group-introduced polypeptide was obtained. On the other hand, 7.9 mg (200 nmoles) of HRP was dissolved in 0.95 ml of 0.02 M phosphate buffer (pH 6.8) containing 0.15 M NaCl, and 50 μl of a DMF solution containing 1.54 mg (4.9 μmoles) of [N-succinimidyl-3-(2-pyridylthio)propionate] (SPDP) was mixed therewith, followed by reaction at room temperature for 40 miuutes. After the reaction, 0.33 ml of 0.1 M acetatc buffer (pH 4.5) containing 8.2 mg (53 μmolcs) of dithiothreitol was added thereto, followed by reaction at room temperature for 20 minutes. Then, the reaction product was fractiouated on a Sephadex G-25 column. Thus, 6 mg (100 nmoles) of a SH group-introduced enzyme was obtained. Then, 100 nmoles of maleimide group-introduced PACAP38NH_2 and 100 nmoles of SH group-introduced HRP werc mixed and reacted with each other at 4° C. for 16

hours. After the reaction, the reaction prodnct was fraction-ated on Ultrogel AcA44 (LKB-Pharmacia) to obtain HRP-labeled PACAP38NH_2.

(2) Measurement of Antibody Titer of Monse Antiserum

[0296] The antibody titer of the mouse antiserum was measured by the following method. In order to prepare an anti-mouse immunoglobulin antibody-bonnd microplate, 100 μl of 0.1 M carbonate buffer (pH 9.6) containing 100 μg/ml of the anti-monse immunoglobnlin antibody [IgG fraction, Kappel] was first poured into each well of a 96-well microplate, and the plate was allowed to stand at 4° C. for 24 hours. After the plate was washed with PBS, 300 μl of PBS containing 25% Blockace (Snow Brand Milk Prodncts) was poured into each well to block excess binding sites of the wells, and treated at a temperature of 4° C. for at least 24 hours. To each well of the above-mentioned anti-monse immunoglobnlin antibody-bound microplate, 50 μl of buffer E [0.02 M phosphate buffer (pH 7.0) containing 10% Block-ace, 2 mg/ml bovine serum albumin (BSA), 0.4 M NaCl, 2 mM EDTA and 0.1% NaN_3] or 50 μl of the mouse anti-PACAP38NH_2 antiserum diluted with buffer E were added, followed by reaction at 4° C. for 16 hours. After the plate washed with PBS, 100 μl of the HRP-labeled PACAP38NH_2 prepared in Example 2(1) described above [diluted 200 times with 0.02M phosphate bnffer (pH 7.0) containing 2 mg/ml BSA and 0.15 M NaCl (buffer H)] was added to each well, followed by reaction at room temperature for 6 honrs. After the reaction, the plate was washed with PBS, and then 100 μl of 0.1 M citrate buffer (pH 5.5) containing 0.2% o-phenylenediamine and 0.02% hydrogen peroxide was poured into each well, followed by reaction at room temperature for 10 minntes in order to measure the enzyme activity on the solid phase. After 100 μl of 4N snlfuric acid was added thereto to terminate the reaction, the absorption at 492 nm was measured by a plate reader (MTP-32, Corona). As a result, increases in anti-PACAP38 antibody titer were observed in 4 mice out of the 8 immunized mice.

EXAMPLE 3

(1) Cell Fusion

[0297] Mouse No. 5, which exhibited a relatively high serum antibody titer in Example 2 above, was snbjected to final immnnization by intravenons injection of 200 μl of immunogen dissolved in 0.25 ml of saline. Four days after the final immunization, the spleen was extirpated from the mouse, compressed and filtered through stainless steel meshes, and suspended in Eagle's minimum essential medium (MEM), to yield a splenocyte snspension. BALB/C mouse-derived myeloma cells P3×63.Ag8.U1 (P3U1) were nsed as a cell fusion partner (*Curr. Topics Microbiol. Immunol.*, 81: 1, 1978). Cell fusion was performed in accordance with an original method (*Nature*, 256: 495, 1957). That is, splenocytes and P3U1 were separately washed with serum-free MEM three times and mixed so that the ratio by number of the splenocytes and P3U1 was 5:1, and the mixed cells were precipitated by a centrifugation at 800 rpm for 15 minutes. After the supernatant was thoroughly removed, the precipitate was gently loosened, and 0.3 ml of 45% poly-ethylene glycol (PEG) 6000 (mannfactured by Koch-light Ltd.) was added, and the mixture was allowed to stand in a warm water chamber at 37° C. for 7 minntes to cause fusion. After the fusion, MEM was added to the cells at a rate of 2 ml per minnte; after a total of 12 ml of MEM was added, the mixture was centrifuged at 600 rpm for 15 minntes, and the snpernatant was removed. This cell precipitate was suspended in a G1T medium (Wako Pure Chemical) containing 10% fetal calf serum (G1T-10FCS) to obtain a P3U1 cell density of 2×10^6 cells per ml, and the suspension was inocnlated to 120 wells of a 24-well multidish (mannfactured by Linbro Company) at 1 ml per well. After the inocnlation, the cells were cnltured in a CO_2 incnbator at 37° C. nnder an atmosphere of 5% CO_2/95% air. After 24 hours, HAT selection cnlture was started by adding a G1T-10FCS medium containing HAT (hypoxanthine 1×10^{-4} M, aminop-terin 4×10^{7} M, thymidine 1.6×10^{-3} M) (HAT mednm) at 1 ml per well. The HAT selection culture was continned by discarding 1 ml of the old mednm and thereafter adding 1 ml of HAT mednm at 3, 6, and 9 days after the start of the cnltivation. Colonies of hybridoma were observed 9 to 14 days after the cell fusion; when the cnlture medium turned to yellow (about 1×10^6 cells/ml of cell density), the super-natant was collected and antibody titer was measnred.

(2) Hybridoma Screening

[0298] Fifty microliter of bnffer E and 50 μl of the hybridoma cnlture snpernatant were added to the above-mentioned anti-mouse immunoglobnlin antibody-bound microplate, and the reaction was carried out at room temperature for 6 hours. After the plate washed with PBS, 100 μl of the HRP-labeled PACAP38NH_2 prepared in Example 2(1) above [dilnted 200-fold with bnffer H] was added, and the reaction was carried ont at 4° C. for 16 hours. Snbse-qnently, after the plate washed with PBS, the enzyme activity on the solid phase was measured by the method described in Example 2(2) above. The antibody titer was observed in 18 wells out of total of 120 wells showing hybridoma growth, when examined as described above.

(3) Cloning

[0299] Hybridomas of No. 44, No. 49, No. 97, and No. 113 wells out of the wells that showed anti-PACAP antibody positive were snbjected to cloning by a limiting dilntion method. That is, hybridomas were suspended in RPMI1640-20FCS to obtain a density of 1.5 cells/ml, and each snspen-sion was dispensed to a 96-well microplate (manufactured by Nunc Company) at 0.2 ml per well. On this occasion, thymocytes isolated from BALB/C monse were added to each well at 5×10^5 cells per well as feeder cells. Abont 1 week later, colony formation was observed, when the anti-body titer in the culture snpernatant was measured by the EIA method described in Example 2(2) above, 28 of the 30 clones of the hybridoma from No. 44 well, 47 of the 50 clones of the hybridoma from No. 49 well, 49 of the 50 clones of the hybridoma from No. 97 well, and 48 of the 50 clones of the hybridoma from No. 113 well prodnced the anti-PACAP antibody.

[0300] Taking note of some of these clones, namely, the clone PA-6N obtained from No. 44-2 and the monoclonal antibody PA-6Na produced thereby, the clone PA-1N obtained from No. 49-3 and the monoclonal antibody PA-1Na produced thereby, the clone PA-2C obtained from No. 97-2 and the monoclonal antibody PA-2Ca produced thereby, and the clone PA-5N obtained from No. 113-5 and the monoclonal antibody PA-5Na produced thereby, the following experiment was performed. Likewise, a cell fusion experiment was performed nsing splenocytes from

another mouse immunized with the same immunogen, and taking note of the clone PA-1C obtained from No. 28-12 and the monoclonal antibody PA-1Ca produced thereby and the clone PA-3N obtained from No. 10-3 and the monoclonal antibody PA-3Na produced thereby, the following experiment was performed.

(4) Purification of Monoclonal Antibodies

[0301] The above-mentioned hybridoma was intraperitoneally injected to mouse (BALB/C) pre-treated with an intraperitoneally administration of mineral oil (0.5 ml) or the untreated mouse at $1\text{-}3\times10^3$ cells/mouse, and ascites containing the antibody were collected 6-20 days later. The monoclonal antibodies were purified from the ascites by using a Protein-A column or a diethylaminoethyl (DEAE)-cellulose column. Namely, 6 ml of the ascites from the mice inoculated with PA-1N was diluted with the same amount of a binding buffer (1.5 M glycine buffer containing 3.5 M NaCl and 0.05% NaN_3 (pH 9.0)). The resulting solution was applied onto a Protein-A Sepharose column (Pharmacia) which had been pre-equilibrated with the binding buffer, and the specific antibody was eluted with an elution buffer (0.1 M citrate buffer containing 0.05% NaN_3 (pH 3.0)). By the above procedures, 28 mg of the specific antibody was obtained. Similarly, 23 mg of a specific antibody was obtained from 5 ml of the ascites from the mice inoculated with PA-5N, 13 mg of a specific antibody was obtained from 7.5 ml of the ascites from the mice inoculated with PA-6N, and 45 mg of a specific antibody was obtained from 14 ml of the ascites from the mice inoculated with PA-1C. On the other hand, a salt precipitation was applied to 20 ml of the ascites from the mice inoculated with PA-3N by adding saturated ammonium sulfate solution to a final concentration of 45%, which was followed by centrifugation (20,000×g, 30 minutes). The precipitate fraction was dialyzed against 0.02 M borate buffer (pH 8) containing 0.15 M NaCl (BBS), and further dialyzed against 0.01 M phosphate buffer containing 0.01 M NaCl. The antibody fraction was applied on a DEAE cellulose column (DE-52, Wattman, 2.5×10 cm), and eluted by a 100 ml of linear concentration gradient (0.01 M-0.35 M) of NaCl. By the above procedures, 136 mg of a specific antibody was obtained. Similarly, 57 mg of a specific antibody was obtained from 7,5-ml of the ascites from the mice inoculated with PA-2C.

EXAMPLE 4

Determination of Class and Subclass of Monoclonal Antibody

[0302] One hundred microliter of 0.1 M carbonate buffer (pH 9.6) containing 5 μg/ml of $PACAP38NH_2$ was poured into each well of a 96-well microplate, and the microplate was allowed to stand at 4° C. for 24 hours. The excess binding sites of the wells were blocked with Blockace according to the method described in Example 5. Thus, a $PACAP38NH_2$-bound plate was prepared. Then, each 100 μl of culture supernatants of PA-1N, PA-3N, PA-5N, PA-6N, PA-2C and PA-1C was added to the plate, followed by reaction at room temperature for 3 hours. Then, the class and subclass were examined by EL1SA using an isotype typing kit (Mouse-Typer™ Sub-1sotyping Kit, Bio RAD). As a result, PA-lNa, PA-6Na, PA-2Ca and PA-lCa belonged to lgGl, κ, PA-5Na belonged to lgG2a, κ and PA-3Na belonged to lgG2b, κ.

EXAMPLE 5

(1) Preparation of $F(ab')_2$ Fraction

[0303] PA-6Na was concentrated to 8 mg/500 μl by a Collodion bag (M&S 1nstruments 1nc.), and then dialyzed against 0.1 M acetate buffer containing 0.1 M NaCl. To the resulting antibody solution was added 0.4 mg of pepsin (crystallized twice, Sigma), followed by reaction at 37° C. for 16 hours. Then, the $F(ab')_2$ fraction was purified by an FPLC (Pharmacia) using a Superose 12 column equilibrated with 0.1 M phosphate buffer (pH 6.8). By a similar method, 0.445 mg of pepsin was added to 8.9 mg of PA-lCa to prepare the $F(ab')_2$ fraction.

(2) Preparation of HRP-Labeled Anti-PACAP Monoclonal Antibodies

[0304] One milliliter of the PA-6Na $F(ab')_2$ fraction [2.2 mg (22 nmole)/ml] was mixed with 50 μl of a DMF solution containing GMBS (260 nmole), followed by reaction at room temperature for 40 minutes. The reaction solution was fractionated on a Sephadex G-25 column [1×30 cm, eluent: 0.1 M-phosphate buffer (pH 6.7)] to obtain 1.5 mg of a maleimide group-introduced $F(ab')_2$ fraction. With 1.5 mg of the maleimidated $F(ab')_2$ fraction was mixed with 5.5 mg of SH group-introduced HRP prepared by the method described in Example 2(1) above, and the reaction product was concentrated to about 0.3 ml by a collodion bag, followed by reaction at 4° C. for 16 hours. The reaction solution was applied on an Ultrogel AcA34 column (10 mmϕ×40 mm) using 0.1 M phosphate buffer (PH 6.5) as an eluent to purify an $F(ab')_2$-HRP complex fraction. It was confirmed that 2.4 molecules of HRP were introduced per $F(ab')_2$ molecule by comparing the absorbance at 280 nm and 403 nm. In a similar manner, an $F(ab')_2$-HRP complex was prepared by using 2.9 mg of the PA-lCa $F(ab')_2$ fraction. Furthermore, 6.4 mg (43 nmoles) of the PA-2Ca purified fraction was maleimidated by adding 15-fold moles of GMBS. Then, the resulting product was reacted with SH group-introduced HRP in a similar manner to prepare the labeled antibody into which HRP was introduced in an amount of 2.4 molecules/molecule of lgG1.

EXAMPLE 6

Investigation of Region in Antigen Recognized by Each Monoclonal Antibody

(1) PA-lNa

[0305] Fifty microliter of a PA-1N culture supernatant diluted 50-fold with buffer H and 50 μl of a buffer H solution containing $PACAP38NH_2$, $PACAP27NH_2$, PACAP(4-27)OH, PACAP(1-13)OH, $PACAP(14\text{-}38)NH_2$, $PACAP(31\text{-}38)NH_2$ or V1P(SEQ 1D NO:7) were added to the anti-mouse immunoglobulin antibody-bound microplate described in Example 2(2) above, followed by reaction at room temperature for 2 hours. Then, 50 μl of HRP-labeled $PACAP38NH_2$ obtained in Example 2(1) above (diluted 100-fold with buffer H) was added thereto, followed by reaction at 4° C. for 16 hours. After the reaction, the plate washed with PBS, and then the enzyme activity on the solid phase was measured by the method described in Example 2(2) above. The results are shown in F1G. 1(*a*). PA-lNa reacted with $PACAP38NH_2$, $PACAP27NH_2$, PACAP(1-13)OH and PACAP(4-27)OH, but did not react with $PACAP(14\text{-}38)NH_2$

and PACAP(31-38)NH_2. PA-lNa did not react with VlP either (the cross reactivity was 0.1% or less (to PACAP38NH_2)). These results reveal that the antibody recognizes the N-terminal portion of PACAP38NH_2.

(2) PA-3Na

[0306] A competitive ElA nsing PA-3Na (50-fold dilntion of a PA-3N culture supernatant) was carried out by the method described in the above (l). The resnlts are shown in FlG. 1(*b*). PA-3Na reacted with PACAP38NH_2, PACAP27NH_2, PACAP(1-13)OH and PACAP(4-27)OH, but did not react with PACAP(14-38)NH_2 and PACAP(31-38)NH_2 (the cross reactivity was 0.1% or less (to PACAP38NH_2)). PA-3Na showed cross reactivity of 1% with VlP (to PACAP38NH_2). These results reveal that the antibody rccognizcs the N-terminal portion of PACAP38NH_2.

(3) PA-5Na

[0307] A competitive ElA using PA-5Na (70-fold dilntion of a PA-5N culture supernatant) was carried ont by the method described in the above (l). The results are shown in FlG. 1(*c*). PA-5Na reacted with PACAP38NH_2, PACAP27NH_2, PACAP(1-13)OH and PACAP(4-27)OH, but did not react with PACAP(14-38)NH_2 and PACAP(31-38)NH_2. PA-5Na did not rcact with VlP either (the eross reaetivity was 0.1% or less (to PACAP38NH_2)). These results reveal that the antibody recognizes the N-terminal portion of PACAP38NH_2. PA-lNa was different from PA-5Na in eross reactivity to PACAP(1-13)OH (in comparison with PACAP38NH_2), and the cross reactivity of the former was at least 10 times stronger than that of the latter.

(4) PA-6Na

[0308] A competitive ElA nsing PA-6Na (40-fold dilution of a PA-6N culture supernatant) was carried out by the method described in thc above (1). The resnlts are shown in FlG. 1(*d*). PA-6Na reacted with PACAP38NH_2, PACAP27NH, and PACAP(4-27)OH, but did not react with PACAP(1-13)OH, PACAP(14-38)NH_2 and PACAP(31-38)NH_2. PA-6Na did not react with VlP either (the cross reactivity was 0.1% or less (to PACAP38NH_2)). These resnlts reveal that thc antibody recognizes the region from the N-tcrminal portion to thc central portion of PACAP38NH_2.

(5) PA-2Ca

[0309] A compctitive ElA using PA-2Ca (340-fold dilution of a PA-2C culture snpernatant) was carried ont by the method dcscribed in the above (1). The resnlts are shown in FlG. 1(*e*). PA-2Ca reacted with PACAP38NH_2 and PACAP(14-38)NH_2, but did not react with PACAP27NH_2, PACAP(4-27)OH PACAP(1-13)OH and PACAP(31-38)NH_2. PA-2Ca did not react with VlP either (the cross reactivity was 0.1% or less to PACAP38NH_2). These resnlts reveal that the antibody recognizes the region from the C-terminal portion to the central portion of PACAP38NH_2.

(6) PA-lCa

[0310] A competitive ElA using PA-lCa (35-fold dilution of a PA-1C culture supernatant) was carricd out by the method described in thc above (1). The resnlts are shown in FlG. 1(*f*). PA-lCa reacted with PACAP38NH_2, PACAP(14-38)NH_2 and PACAP(31-38)NH_2, bnt did not react with PACAP27NH_2, PACAP(4-27)OH and PACAP(1-13)OH. PA-1Ca did not react with VlP either (the cross reactivity was 0.1% or less (to PACAP38NH_2)). These results reveal that the antibody recognizes the C-terminal portion of PACAP38NH_2.

[0311] Based on the resnlts shown above, the region in antigen recognized by the above-described six kinds of anti-PACAP monoclonal antibodies are snmmarized in FlG. 2.

EXAMPLE 7

Evaluation of Neutralizing Activities of Anti-Pacap Monoclonal Antibodies

[0312] Rat adrenal pheochromocytoma cell line, PC-12h [supplied by Dr. Hatanaka (then) of the Institute for Protein Research, Osaka University; Brain Res., 222(2): 225-33, 1981] were seeded into a collagen-coated 48-well mnltiwell plate (mannfactured by Sumitomo Bakelite Co., Ltd.) at 5×10^4 cells/well, and cultured in Dnlbecco's modified Eagle's medium (DMEM) containing 10% FCS for 7 to 10 days. The medium in the plate was replaced with Hanks' balanced salt solntion (HBSS) containing 0.05% BSA, and the cells were eultured for 30 minntes. Then, PACAP38NH_2 (final concentration 2 nM) that had been previously reacted with each of the above-described six kinds of anti-PACAP monoclonal antibodies (final concentration 2, 20 or 200 nM) at 4° C. for 1 hour, was added to the plate. After the cells were fnrther cultured for 2 hours, the cAMP concentration in the cnlture snpernatant was measnred nsing a cAMP assay kit (mannfactured by Amersham Company). The results are shown in FlG. 3. It was fonnd that two antibodies (PA-6Na and PA-1Ca) out of the six kinds of anti-PACAP monoclonal antibodies had no neutralizing activity against PACAP38NH_2.

EXAMPLE 8

Evaluation of Suppressive Effect of Anti-PACAP38 Non-Nentralizing Antibodies on Pacap Degradation by DPP-lV

(1) Snppressive Effect on PACAP Degradation by Endogenons DPP-lV from CaCo-2 Cells

[0313] Whethcr or not anti-PACAP38 non-nentralizing antibodies snppress PACAP degradation by DPP-lV was investigated nsing DPP-lV (dipeptidyl peptidase lV) expressed in CaCo-2 cells (human colon adenocarcinoma-derived cells).

[0314] After a CaCo-2 cell membrane fraction was placed in Tris-EDTA-CHAPS bnffer [20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 0.1% BSA and 0.05% CHAPS] and diluted stepwise, PACAP38 was added at a final concentration of 1.0×10^{-8} M in the presence or absence of 1.3×10^{-7} M of PA-6Na, and the mixture was incubated at 37° C. for 4.5 hours (expcriment 1). To evalnate the nonselective adsorption of PACAP38 to the CaCo-2 cell membrane fraction, 4×10^{-7} M of a DPP-lV inhibitor (IC_{50}=5 nM) was added in advance, and the mixture was incnbated under the same conditions as experiment 1 (experiment 2). After 4×10^{-7} M of the above-described DPP-lV inhibitor was added to the sample of experiment 1 to stop the reaction, the reaction mixtures of expcriments 1 and 2 were dilnted

10-fold with Tris-EDTA-Digitonin buffer [20 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 0.1% BSA, 0.05% Digitonin, 0.5 mM PMSF, 10 μg/ml Pepstatin A, 20 μg/ml Leupeptin, and 4 μg/ml E-64], and 100 pM of a PACAP receptor [purified using the method described in Ohtaki, T. et al.: *J Biol Chem* 273, 15464-73. (1998) with partial modification] and 100 pM of ^{125}I-PACAP27 (Ohtaki T et al. *Biochem. Biophys. Res. Commun.* 171, 838-844) were added thereto. After incubation at 25° C. for 75 minutes, the reaction mixture was applied to suction filtration using a polyethylenimine-treated GF/F glass filter (Whatman), and the radioactivity of the ^{125}I-PACAP27 trapped on the filter was detected using a Y counter. The measurements were performed in triplicate. The results are shown in FIG. **4**. The stronger residual radioactivity shows larger amount of ^{125}I-PACAP27 bound to the receptor, indicating decrease in the residual amount of the previously added PACAP38 in sample. From this Figure, it was found that DPP-IV exhibited concentration-dependent PACAP38 degradation in the absence of PA-6Na, whereas PA-6Na nearly completely suppressed the degradation of PACAP38 in the range of DPP-IV concentrations examined.

(2) Suppressive Effect on Degradation by a Recombinant DPP-IV

[0315] The same kind of experiment was performed under the same reaction conditions as with the use of CaCo-2 but using semi-purified recombinant DPP-IV in place of the CaCo-2 membrane fraction. The use of the recombinant DPP-IV lessened the non-selective binding, which was problematic during the evaluation of the PACAP38 degradation with the CaCo-2 membrane fraction, and improved the substrate degradation activity (FIG. **5**, —□—).

[0316] Irrespective of the presence or absence of a DPP-IV inhibitor, PA-6Na suppressed the degradation of PACAP38 by DPP-IV in the DPP-IV concentration range from 1- to 100-fold dilution (FIG. **5**, —●— and —○—). That is, a suppression effect of PA-6Na on PACAP38 degradation by DPP-IV was clearly detected without previously adding the DPP-IV inhibitor.

EXAMPLE 9

Preparation of an Immunogen Comprising GLP-1(7-36) Amide

[0317] Four milligram of GLP-1(7-36)amide (manufactured by AnaSpec Company) and 7.2 mg of BSA were dissolved in 2 ml of 0.1M phosphate buffer (pH 6.8), and 2 ml of glutaraldehyde, previously diluted to 0.1% in 0.1M phosphate buffer (pH 6.8) was gently added thereto drop by drop, and the reaction was carried out under ice cooling for 5 hours. After the reaction mixture was dialyzed against calcium- and magnesium-free Dulbecco's phosphate buffered saline (D-PBS(−)), the dialysate was preserved in divided portions at −80° C., and this was used as the immunogen.

EXAMPLE 10

Measurement of Anti-GLP-1 Antibody Concentrations (Enzyme Immunoassay)

[0318] An anti-mouse immunoglobulin goat antibody (IgG fraction, manufactured by Jackson ImmunoResearch Laboratories Inc.) was diluted to a concentration of 5 μg/ml in 0.01 M phosphate buffer (pH 8.0) containing 0.01 M NaCl, this was dispensed to a 96-well half-area plate (manufactured by Corning Inc.) at 50 μl per well, and the antibody was adsorbed at 4° C. overnight. After this solution was removed, a D-PBS(−) containing 25% BlockAce (manufactured by Dainippon Pharmaceutical Co., Ltd.) was added at 100 μl per well, and the plate was allowed to stand at 37° C. for 1 hour.

[0319] After this plate washed with a D-PBS containing 0.05% Tween 20 (PBS-T), 50 μl portion of an antiserum, previously serially diluted with a D-PBS containing 0.1% Tween 20, was added, and the reaction was carried out at 37° C. for 1 hour. After the plate washed with PBS-T, a biotin-labeled GLP-1 [GLP-1(7-36)-Lys(Biotin), amide] (manufactured by AnaSpec Inc.), previously diluted to 10 ng/ml with D-PBS(−) containing 0.1% Tween 20, was dispensed at 50 μl per well, and the reaction was carried out at 37° C. for 1 hour. After the plate washed with PBS-T, horseradish peroxidase (HRP)-labeled streptavidin (manufactured by Jackson ImmunoResearch Laboratories Inc.), previously diluted to 0.3 μg/ml with D-PBS(−) containing 0.1% Tween, was dispensed at 50 μl per well, and the reaction was carried out at room temperature for 30 minutes. After the plate was further washed with PBS-T, an HRP substrate (TMB Peroxidase EIA Substrate Kit, manufactured by Bio-Rad Laboratories, Inc.) was dispensed at 50 μl per well to develop a color; after 1 N sulfuric acid was dispensed at 50 μl per well to stop the reaction, absorbance at 450 nm was measured using a plate reader (Multiscan; Labosystems Japan Co.).

EXAMPLE 11

[0320] (1) Immunization with GLP-1(7-36)amide Complex

[0321] Ten BALB/c mice (13-week-old, female) were subcutaneously immunized with an emulsion prepared by mixing the GLP-1(7-36)amide/BSA complex solution prepared in Example 9 and Freund's complete adjuvant in a 1:1 ratio, by volume at 50 μg/animal. In the second immunization and thereafter, an emulsion prepared with the GLP-1(7-36)amide/BSA complex and Freund's incomplete adjuvant was given at 2-week intervals. One week after the third immunization, final immunization was performed by intravenously administering the above-described immunogen dissolved in 100 μl of saline to individuals showing high serum antibody titers in the ELISA described in Example 10.

(2) Obtainment of Anti-GLP-1 Antibody-Producing Hybridomas

[0322] Three days after the final immunization, the spleen was extirpated from the mouse, and about 10^8 splenocytes were obtained via centrifugal washing procedure. After the splenocytes and mouse myeloma cells P3X63.Ag8.U1 (P3U1) were mixed at a 5:1 cell count ratio, the cells were fused by using polyethylene glycol 1500 (manufactured by Roche Diagnostics) in accordance with the method of Kohler and Milstein (Nature, 256: 495, 1957). The fused cells were resuspended in an IH medium [a 1:1 mixed medium of IMDM (manufactured by Invitrogen Co.) and Ham's F-12 (manufactured by Invitrogen Co.)] containing 10% fetal bovine serum (IH-FCS), and seeded into a 96-well multiple well plate (manufactured by Corning Inc.) at 2×10^4 cells/100 μl/well based on P3U1 cell, and cultured in a CO_2

incubator at 37° C. nnder 5% CO_2 atmosphere overnight. The following day, an 1H-FCS containing hypoxanthine, aminopterin and thymidine (HAT medium) was added to each well at 100 μl/well, and selection cnlture was performed in a CO_2 incnbator at 37° C. nnder 5% CO_2 atmosphere. For the HAT selection culture, ¾ of the cnlture supernatant from each well was exchanged with a fresh HAT medium 4 days and 7 days after the cell fusion, and the culture was continued. The cnlture snpernatants from the wells showing hybridoma growth from 8 days to 2 weeks after the cell fusion were snbjected to the ELISA described in Example 10 and 15 kinds of anti-GLP-1(7-36) monoclonal antibody-producing hybridomas [GLIT1-175(1)18, GLIT1-238(1)6, GLIT1-254(1)6, GLIT1-256(1)31, GLIT1-464(1)15, GLIT1-492(1)2, GLIT2-105(1)6, GLIT2-130(1)36, GLIT2-197(1)66, GLIT2-234(2)65, GLIT2-329(1)24, GLIT2-357(1)10, GLIT2-806(1)4, GLIT2-851(1)11, GLIT2-863(1)35] were obtained, and cloned cell lincs wcrc cstablishcd by a limiting dilution mcthod.

(3) Preparation of Pnrified Anti-GLP-1 Monoclonal Antibodies

[0323] The 15 kinds of anti-GLP-1 monoclonal antibody-producing hybridomas obtained above were cultured in an 1H medium containing 10% Ultra low lgG FCS (manufactured by lnvitrogen Co.) in a CO_2 incubator at 37° C. nnder 5% CO_2 atmosphere, and the culture snpernatants were collected by centrifugation and subjected to Protein A colnmn chromatography to yield pnrified antibodies. The snbclasses of these purified antibodies were determined using the lsoStrip Mouse Monoclonal Antibody lsotyping Kit (manufactured by Roche Diagnostics). These anti-GLP-1 antibodies exhibited high binding activities of EC_{50}: 1×10^{-9} to 1×10^{-11} M, against a biotin-labeled GLP-1(7-36)amide, in the ELISA described in Example 10. Also, in the same ELISA, a given concentration of each of the varions anti-GLP-1 antibodies was trapped onto an anti-monse immnnoglobulin goat antibody-coated plate, after which a given concentration (10 ng/mL) of a biotin-labeled GLP-1(7-36)amide and a 0.003- to 300-fold molar amount of GLP-1(7-36)amidc (manufactured by AnaSpec Company) or GLP-1(9-36) amide (manufactured by Phoenix Pharmaceuticals Company) were added, and the binding inhibition of the labeled GLP-1(7-36)amide were examined; as a resnlt, all antibodies were found to be antibodies specifically recognizing the N-terminal region of GLP-1, which were strongly inhibited by GLP-1(7-36)amide bnt were not inhibited by GLP-1(9-36)amide. The above resnlts are snmmarized in Table 1.

TABLE 1

Biotin-labeled GLP-1(7-36) amide binding inhibitory activities (IC_{50}) of various anti-GLP-1 antibodies

Name of antibody clone	Biotin-labeled GLP-1(7-36) amide binding inhibitory activity (IC50 (M)) GLP-1(7-36) amide	GLP-1(9-36) amide
GLIT1-175(1)18	2.5E-09	>1.4E-07
GLIT1-238(1)6	1.9E-09	>1.4E-07
GLIT1-254(1)6	1.4E-09	5.1E-07
GLIT1-256(1)31	2.4E-09	>1.4E-07
GLIT1-464(1)15	1.6E-09	>1.4E-07
GLIT1-492(1)2	1.5E-09	5.8E-07

TABLE 1-continued

Biotin-labeled GLP-1(7-36) amide binding inhibitory activities (IC_{50}) of various anti-GLP-1 antibodies

Name of antibody clone	Biotin-labeled GLP-1(7-36) amide binding inhibitory activity (IC50 (M)) GLP-1(7-36) amide	GLP-1(9-36) amide
GLIT2-105(1)6	2.4E-09	>1.4E-07
GLIT2-130(1)36	2.0E-09	>1.4E-07
GLIT2-197(1)66	4.0E-09	>1.4E-07
GLIT2-234(2)65	3.0E-09	>1.4E-07
GLIT2-329(1)24	3.6E-09	2.1E-06
GLIT2-357(1)10	5.8E-09	>1.4E-07
GLIT2-806(1)4	9.0E-09	7.6E-07
GLIT2-851(1)11	2.0E-09	>1.4E-07
GLIT2-863(1)35	2.2E-09	>1.4E-07

EXAMPLE 12

Selection of Anti-GLP-1 Non-Nentralizing Monoclonal Antibody

[0324] To select a non-neutralizing antibody that does not inhibit the binding of GLP-1 to GLP-1 receptor, out of the 15 kinds of anti-GLP-1 monoclonal antibodies prepared in Example 11, the following two kinds of assays measuring GLP-1 activity were performed.

(1) GLP-1 Receptor Reporter Gene Assay

[0325] A CHO cell line stably expressing GLP-1 receptor on the cell membrane surface and transfected with the Mnltiple Response Element (MRE)/cAMP Response Element (CRE)-lucifcrase gene, werc suspcnded in Ham's F-12 medium (manufactured by lnvitrogen Co.) containing 10% fetal bovine serum, 1 μg/ml blastocidin, 500 μg/ml geneticin, and 50 mg/ml gentamycin, and the cell suspension was seeded into a 96-well white plate (manufactured by Corning Coster Co.) at 1×10^4 cells/well, and cnltured nsing a CO_2 incnbator at 37° C. nnder 5% CO_2 atmosphere for 2 days. After the mcdium was removed by aspiration, 100 μl of a reaction mixture of GLP-1(7-36)amide (2 nM) and a 1- to 300-fold molar amount of pnrified anti-GLP-1 monoclonal antibody[dilnted with Ham's F-12 (mannfactured by lnvitrogen Co.)], pre-incnbated at room temperature for 1 honr, was added, and the cells were cnltured in a CO_2 incubator at 37° C. under 5% CO_2 atmosphere for 5.5 hours. After the medium was removed by aspiration, a 1:1 mixture of PicaGene LT-FR (manufactured by Toyo Ink MFG Co., Ltd.) and Hanks' balanced salt solntion (HBSS) was added to each well at 40 μl/well. The reaction was carried ont at room temperature for 20 minntes, after which transcriptional activity on MRE/CRE was qnantified with lnciferase activity as the index, by using the Wallac 1420 ARVOMX Multilabel connter (mannfactured by PerkinElmer Japan Company).

(2) GLP-1/GLP-1 Receptor Binding Inhibition Assay

[0326] A cell membrane fraction wherein the GLP-1 receptor was overexpressed was prepared as described below. That is, a recombinant baculovirus solntion was prepared from the pFastBac™ piasmid (mannfactured by Invitrogen Lifetechnologies Company) inserted with the human GLP-1 receptor gene by a conventional method, and the virus was infected to an insect cell line (Sf9) to tran-

siently express the GLP-1 receptor. These infected cells were collected by a centrifugation and disrupted with Polytron in the presence of a surfactant, after which the cell debris was removed by a centrifugation at 600×g, and the supernatant was ultracentrifuged at 100,000×g to yield a membrane fraction containing the GLP-1 receptor.

[0327] Two hundreds picomolar of ^{125}I-labeled GLP-1 (IM323, manufactured by Amersham Bioscience) and a 1- to 1,000-fold molar amount of anti-GLP-1 antibody were mixed and incubated at 37° C. for 1 hour, after which the above-described GLP-1 receptor membrane fraction was added, and the mixture was incubated at room temperature for 75 minutes, after which B/F separation was performed using a polyethylenimine-treated glass filter (GF/F, manufactured by Watman), and the amount of ^{125}I-labeled GLP-1 bound to the GLP-1 receptor was quantified using a γ counter.

[0328] The results of the above experiment (1) and (2) are summarized in Table 2. Antibodies that did not inhibit the binding of GLP-1 to GLP-1 receptor even when added at a 1,000-fold molar amount to GLP-1(7-36)amide, and that did not neutralize GLP-1 activity in the GLP-1 receptor reporter gene assay even when added at a 300-fold molar amount (see FIG. 6 and FIG. 7) were designated as anti-GLP-1 non-neutralizing antibodies. Out of the two kinds of anti-GLP-1 non-neutralizing antibodies obtained (Table 2), GLIT2-329(1)24, which showed better physical stability, was selected as the anti-GLP-1 non-neutralizing antibody and subjected to the experiment described below.

TABLE 2

Results of GLP-1 receptor reporter gene assay and GLP-1/GLP-1 receptor binding inhibition assay of anti-GLP-1 antibodies

Name of antibody clone	GLP-1 receptor reporter gene assay	GLP-1/GLP-1 receptor binding inhibition assay
GLIT1-175(1)18	−	++
GLIT1-238(1)6	−	++
GLIT1-254(1)6	+++	+++
GLIT1-256(1)31	++	+
GLIT1-464(1)15	+++	binds to GF
GLIT1-492(1)2	++	+++
GLIT2-105(1)6	−	+
GLIT2-130(1)36	−	+
GLIT2-197(1)66	−	+
GLIT2-234(2)65	−	+
GLIT2-329(1)24	−	−
GLIT2-357(1)10	−	+
GLIT2-806(1)4	−	−
GLIT2-851(1)11	−	+
GLIT2-863(1)35	+	+++

+: Positive for neutralization activity,
−: Negative for neutralization activity,
GF: Glass filter

EXAMPLE 13

Suppressive Effect of Anti-GLP-1 Non-Neutralizing Monoclonal Antibody (GLIT2-329(1)24) on GLP-1(7-36)amide Degradation by DPP-IV

[0329] The suppressive effect of the anti-GLP-1 non-neutralizing monoclonal antibody (GLIT2-329(1)24) selected in Example 12 on GLP-1(7-36)amide degradation by DPP-IV was examined by ELISA. GLP-1(7-36)amide (final concentration 10 μg/ml) was added to a PBS-T-diluted mixture of anti-GLP-1 monoclonal antibody (GLIT2-329(1)24; final concentration 5 mg/ml) and Dipeptidyl peptidase IV (DPP-IV)(R&D Systems Company; final concentration 200 ng/ml), and the reaction was carried out at 37° C. for 12 hours. After thermally treated at 95° C. for 5 minutes, the reaction mixture was centrifuged at 1800 rpm, and the residual amount of GLP-1(7-36)amide in the supernatant was measured by the method described below. An anti-GLP-1 antibody (GLIT1-254(1)$_6$)that binds to the N-terminal region of GLP-1(7-36)amide but does not bind to GLP-1(9-36)amide was diluted to a concentration of 0.15 μg/ml in D-PBS(−), this was dispensed to a 96-well half-area plate (manufactured by Corning Company) at 50 μl per well, and the antibody was adsorbed at 4° C. overnight. After the solution was removed, 100 μl of a D-PBS(−) containing 25% BlockAce (manufactured by Dainippon Pharmaceutical Co., Ltd.) was added to each well, and the plate was allowed to stand at room temperature for 1 hour. After the plate washed with PBS-T, the above-mentioned reaction mixture, previously serially diluted with a D-PBS(−) containing 0.1% Tween 20, and biotin-labeled GLP-1(7-36)amide, previously diluted to 5 ng/ml with a D-PBS(−) containing 0.1% Tween 20, were dispensed each at 25 μl per well, and the reaction was carried out at 37° C. for 2 hours. After the plate was washed with PBS-T, HRP-labeled streptavidin (manufactured by Jackson ImmunoResearch Laboratories Inc.), previously diluted to 0.3 μg/ml with a D-PBS(−) containing 0.1% Tween, was dispensed at 50 μl per well, and the reaction was carried out at room temperature for 30 minutes. After the plate was further washed with PBS-T, an HRP substrate (TMB Peroxidase EIA Substrate Kit, manufactured by Bio-Rad Laboratories Inc.) was dispensed at 50 μl per well to develop a color, and 1 N sulfuric acid was dispensed at 50 μl per well to stop the reaction, after which absorbance at 450 nm was measured using a plate reader (Multiscan; Labosystems Japan Co.).

[0330] In the absence of antibody (None) and in the presence of anti-erythropoietin antibody (Anti-EPO Ab), More than 90% of GLP-1(7-36)amide was degraded by DPP-IV; whereas in the presence of anti-GLP-1 non-neutralizing monoclonal antibody (GLIT2-329(1)24), only about 10% of GLP-1(7-36)amide was degraded; it was found that this anti-GLP-1 non-neutralizing monoclonal antibody is capable of suppressing the degradation of GLP-1(7-36)amide by DPP-IV (FIG. 8).

EXAMPLE 14

In Vivo Administration Experiment of Anti-GLP-1 Non-Neutralizing Monoclonal Antibody

(1) Administration of Antibody into Rat Peritoneal Cavity

[0331] Saline, an anti-GLP-1 non-neutralizing monoclonal antibody (GLIT2-329(1)24) or control mouse IgG (ChromoPure mouse IgG, whole molecule; manufactured by Jackson ImmunoResearch Laboratories Inc.) was intraperitoneally administered to Wister fatty rats (female) in satiated state at 20 mg/kg: 1 day later, whole blood was drawn from the abdominal aorta, and a DPP-IV inhibitor (DPP4, LINCO Research) was immediately added thereto. Plasma was prepared by centrifugation and cryopreserved.

(2) Measurement of Rat Plasma GLP-1(7-36)amide Concentrations

[0332] The rat plasma concentration of GLP-1(7-36)amide was measured using a GLP-1(7-36)amide assay kit (manufactured by Linco Company) after the pretreatment described below. That is, to a 0.75-mL plasma sample taken from each rat in each administration group described above, an equal volume of Buffer A (1% TFA in 99% distilled water) was added. After the reaction at 4° C. for 5 minutes, it was centrifuged and the supernatant was collected. The supernatant was passed through a C18 column (Sep-column containing 200 mg of C18, manufactured by Peninsula Laboratories Inc.) equilibrated with Buffer A, and the column washed with Buffer A, followed by elution with Buffer B (60% acetonitrile and 1% TFA in 39% distilled water). The eluate was concentrated to dryness under reduced pressure, the residue was dissolved again in 375 μL of the Assay Buffer attached to the above-described ELISA kit, and thermally treated (95° C., 5 minutes), after which it was centrifuged, and the supernatant was subjected to the above-described ELISA kit to measure the GLP-1(7-36)amide concentration. According to the results of an addition-recovery experiment wherein GLP-1(7-36)amide was added to rat plasma and the GLP-1(7-36)amide concentration was measured after the same pretreatment as abovementioned, the recovery of GLP-1(7-36)amide through the pretreatment was 30%. Rat plasma GLP-1(7-36)amide concentrations as corrected by this value are shown in FIG. 9.

[0333] The plasma concentration of active form GLP-1(7-36) in saline-administered satiated Wister Fatty rats at 1 day after administration was 4.7±0.6 pM, and that of active form GLP-1(7-36) in the mouse IgG administration group was 7.1±1.2 pM. On the other hand, the plasma concentration of active form GLP-1(7-36) in the anti-GLP-1 non-neutralizing antibody (GLIT2-329(1)24) administration group was 14.1±4.0 pM, the concentration significantly higher than that of in the mouse IgG administration group as the control ($p<0.05$). This result shows that it is possible to extend the blood half-life of active form GLP-1(7-36) in a living rat, and to raise the blood level thereof by administering the anti-GLP-1 non-neutralizing antibody.

[0334] According to a report describing pre-administration of a DPP-IV inhibitor to an experimental system wherein Zucker fatty rats were loaded with glucose administered from the duodenum, the maximum plasma concentration of active form GLP-1 was about 10 pM at the time when the compound promoted insulin secretion and suppressed blood glucose elevation (Diabetes, 51, 1461-1469, 2002). From this result, it is considered that the active form GLP-1(7-36) concentration observed in the anti-GLP-1 non-neutralizing antibody (GLIT2-329$(1)_{24}$) administration group in this Example, 14.1±4.0 pM, reached in its pharmacologically effective range.

INDUSTRIAL APPLICABILITY

[0335] When the agent for improving the blood stability of the present invention is administered to an animal, the non-neutralizing antibody as the active ingredient thereof binds to an endogenous ligand and stabilizes the same, to raise the blood ligand concentration and/or extend the blood half-life of the ligand, and hence to enhance the receptor activity-regulatory action of the ligand; therefore, the agent of the present invention is useful for the prophylaxis and treatment for a disease involved by an abnormality in the activity of the receptor. In particular, the agent for improving the blood stability of the present invention is useful for a disease involved by an endogenous ligand having a very short blood half-life, such as a peptidic compound, or a disease involved by a receptor for which no agonist is easy to obtain, and the like.

[0336] Also, because the agent for improving the blood stability of the present invention exhibits its effect at an amount of antibody sufficient to obtain the desired blood ligand concentration, it enables a remarkable reduction in clinical dose compared with existing antibody medicines and enables the provision of a safer and inexpensive preparation. Therefore, the agent for improving the blood stability of the present invention contributes to medical cost reductions and significantly contributes to the expansion of the coverage of indications of antibody medicines.

[0337] This application is based on a patent application No. 2004-098595 filed in Japan (filing date: Mar. 30, 2004), the contents of which are incorporated in full herein by this reference.

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20 25

1. An method for improving the blood stability of an endogenous ligand in a mammal, which comprises administering to the mammal an effective amount of an antibody that has an affinity to the endogenous ligand but does not neutralize the same substantially.

2. The method of claim 1, wherein the improved blood stability of the endogenous ligand results in the enhancement of receptor activity-regulatory action thereof.

3. The method of claim 1, wherein the nentralizing activity of the antibody is abont 80% or less.

4. The method of claim 1, wherein the blood concentration of the endogenous ligand becomes abont twice or more compared to the case where the antibody is not administered.

5. The method of claim 1, wherein the blood half-life of the complex of the endogenous ligand and the antibody is about twice or more as that of the endogenous ligand alone.

6. The method of claim 1, wherein the blood half-life of the free endogenons ligand is about one week or less.

7. The method of claim 1, wherein the endogenons ligand is a peptidic componnd.

8. The method of claim 7, wherein the endogenons ligand is one against a G protein-coupled receptor.

9. The method of claim 8, wherein the endogenons ligand is one belonging to secretin/glncagon super family.

10. The method of claim 9, wherein the endogenons ligand is selected from the gronp consisting of GLP-1, calcitonin, PACAP, VIP and analogs thereof.

11. The method of claim 8, wherein the endogenous ligand is selected from the gronp consisting of LHRH, metastin, GPR7/GPR8 ligand, MSH, ghrelin, apelin and analogs thereof.

12. The method of claim 7, wherein the endogenous ligand is selected from the gronp consisting of EPO, TPO, insnlin, interferon, growth hormone, GM-CSF, leptin, adiponectin and analogs thereof.

13. The method of claim 7, wherein the endogenous ligand is selected from the group consisting of ANP, BNP, CNP, betacellulin, betacellulin-δ4, adrenomednllin and analogs thereof.

14. The method of claim 1, which is for the prophylaxis and/or treatment of a disease in which an increased blood concentration and/or a prolonged blood half-life of the endogenous ligand are/is effective for the prophylaxis and/or treatment thereof.

15. The method of claim 14, wherein the disease is selected from the group consisting of metabolic disease, bone and joint disease, cardiovascnlar disease, cranial nerve disease, infectious disease, cancer, blood disorder, urologic disease, infertility/erectile dysfunction, deficient growth and immunodeficiency.

16. A method for the prophylaxis and/or treatment of a disease in a mammal, wherein an increased blood concentration and/or a prolonged blood half-life of an endogenous ligand are/is effective for the prophylaxis and/or treatment of the disease, which method comprises administering to the mammal an effective amonnt of an antibody that has an affinity to the endogenous ligand but does not neutralize the same substantially, without administering a compound the same as or substantially the same as the endogenous ligand, so as to increase the blood stability of the endogenous

ligand, thereby enhancing a receptor activity-regulatory action.

17. A use of an antibody that has an affinity for an endogenous ligand but does not neutralize the same substantially for the manufacture of an agent for the prophylaxis and/or treatment of a disease in which an increased blood concentration and/or a prolonged blood half-life of the endogenous ligand are/is effective for the prophylaxis and/or treatment thereof.

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